

REMARKS

Claims 21 and 28-32 are pending. Claims 28 and 29, due to a restriction requirement, are withdrawn from consideration. Claims 21 and 30-32 are rejected under 35 U.S.C. § 112, first paragraph, and claim 21 is objected to because of a minor informality. Applicants address each basis for rejection as follows.

Claim amendments

Claim 21 has been amended to recite the correct spelling of “erythropoietin” and “erythropoiesis.” Also, the Markush groupings in claim 21 have been amended to list only “and” instead of “and” and “or.”

The subject matter of claim 31 has been incorporated into claim 21. Consequently, claim 31 has been cancelled.

In claim 21, the following amendments have been made:

The phrase “naturally occurring erythropoietin” has been replaced with “wild type erythropoietin.” Support for this amendment is found, for instance, in the Examples section of the specification, where recombinant (wild type) erythropoietin (EPO) is administered.

A “glycosylation variant” of EPO has been replaced with “erythropoietin with a modified N-linked glycosylation pattern” and the claim now recites epoetin and darbepoetin. Support for this amendment is found, for example, at page 12, lines 18-21, of the WO 2005/007183 publication. Here, the specification teaches:

Well described erythropoietin analogues are the hyperglycosylated recombinant proteins epoetin and darbepoetin (or novel erythropoiesis stimulating protein, NESP), of which the structures differ from naturally occurring Epo only by the number of N-linked oligosaccharide[s] on the protein.

In addition, at page 13, lines 3-4, of the WO 2005/007183 publication, the specification states:

US patent application 2003/0077753 describes EPO variants with modified glycosylation patterns.

Recitation of "a point mutant" of EPO has been replaced with "erythropoietin with a point mutation in the helices or interhelical regions of the four alpha helical bundle motif." Also, the claims now recite deletion mutants of "erythropoietin in the helices or interhelical regions of the four alpha helical bundle motif." Support for these amendments is found in the specification, for example, at page 12, lines 24-28, of the WO 2005/007183 publication. Here, the specification states:

The expression product of deletion mutants of a synthetic human Epo cDNA, wherein the point mutations and small deletions in helices and interhelical regions of the four alpha helical [sic] bundle motif have been shown to display biological activity (Bittorf *et al.* (1993) *FEBS Lett* **336**, 133-136; Boissel *et al.* (1993) *J. Biol. Chem.* **268**, 15983-15993).

The point mutations are listed separate from the deletion mutants and the text "stimulates the production of red blood cells" has been repeated. The phrase "stimulates the production of red blood cells" finds support, for example, at page 12, lines 14-16, of the WO 2005/007183 publication. Here, the specification states:

Erythropoietin or Epo as used herein refers to the naturally occurring human cytokine, produced primarily in the kidney which stimulates the production of red blood cells.

The wording of the physiologically tolerated salts of EPO and EPO variants has been adapted to correctly refer to their antecedents.

The phrase "said Gas6 fragment" after "identity at the amino acid level" has been deleted and replaced with "compared to the wild type Gas6 protein." Support for this

amendment can be found, for example, at page 11, lines 3-7, of the WO 2005/007183 publication, where the specification states:

As used herein "mutants of Gas6" refers to modified Gas6 proteins of equal length as wild type Gas6 but with one or more modified amino acids. The sequence homology of such modified Gas6 proteins have a homology of 95% or greater, or 98% or greater or 99% or greater compared to the wildtype Gas6.

As the different Gas6 fragments are now listed in separate clauses (due to removal of the "or" in between), the wording in the physiologically tolerated salts has been changed to provide proper antecedent basis.

No new matter has been added by the present amendments.

Applicants reserve the right to pursue any cancelled subject matter in this or a continuing application.

Rejection under 35 U.S.C. §112, first paragraph

Written Description

Claims 21 and 30-32 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The Office asserts that claim 21 is a genus claim. This claim, according to the Office, is not considered to place limits on the number of alterations that may be made to Gas6 and EPO, and, because the genus is highly variant, recitation of "Gas6 and Epo is insufficient to describe the genus."

Applicants submit that the claims as currently amended encompass variants of Gas6 and EPO that one skilled in the art would recognize as being in the possession of Applicants at the time of filing. As explained below, Applicants submit that the specification provides an adequate written description of the compounds recited in the present claims.

The term "glycosylation variants" has been deleted and the claim now recites epoetin and darbepoetin (both listed in the application as filed, for example, at page 12, line 19), as well as "erythropoietin with a modified N-linked glycosylation pattern." Erythropoietin with a modified N-linked glycosylation pattern was known in the art at the time of filing, as is evidenced by US2003/0077753 cited in the application (see application, page 13, lines 3-4; a copy of US2003/0077753 is enclosed).

Further, the recitation of point mutations in erythropoietin has been restricted to those in the helices or interhelical regions of the four alpha helical bundle motif, where the mutant erythropoietin stimulates the production of red blood cells. Likewise, the deletion mutants of erythropoietin recited in the claims are limited to those in the helices or interhelical regions of the four alpha helical bundle motif, where the mutant erythropoietin stimulates the production of red blood cells. The specification, at page 12, lines 25-28, of the WO 2005/007183 publication states:

[P]oint mutations and small deletions in helices and interhelical regions of the four alpha helical [sic] bundle motif have been shown to display biological activity (Bittorf *et al.* (1993) *FEBS Lett* **336**, 133-136; Boissel *et al.* (1993) *J. Biol. Chem.* **268**, 15983-15993).

Clearly, as is evidenced by reference to two journal articles in the specification, point mutations and deletion mutants in the helices or interhelical regions of the four alpha helical bundle motif that retain the biological activity of EPO (i.e., stimulate the production of red blood cells) were known in the art at the time of filing.

Turning to the Gas6 fragments recited in the claims, Applicants submit that no listing of particular Gas6 fragment or mutants need be recited in the claims. The Office states (page 4):

[I]f activity X is as a ligand (i.e. Gas6 and EPO), and there is no disclosure of the domain(s) responsible for the ligand activity (thus resulting in a synergistic rescue effect on erythropoiesis), the absence of information may be persuasive that those of skill in the art would not take the disclosure as

generic. Accordingly, one of skill in the art would not accept the disclosure of Gas6 and EPO as representative of other proteins having the claimed activity.

Applicants respectfully disagree.

At the time of filing, the domains responsible for the ligand activity of Gas6 were well known to the skilled person. From the specification, it is clear that Gas6 has been isolated and structurally characterized as early as 1993, as is evident from the following passages at page 3 of the WO 2005/007183 publication:

Gas6, the product of the growth arrest-specific gene 6 (*gas6*), is a new member of the vitamin K-dependent protein family (Manfioletti *et al.* (1993) *Mol Cell Biol.* **13**, 4976-4985; Schneider *et al.* (1988) *Cell* **54**, 787-793). (page 3, lines 14-16).

Gas6 is structurally similar to protein S, but lacks a loop, crucial for the anticoagulant activity of protein S (Manfioletti *et al.* cited above). (page 3, lines 22-24).

Applicants also note that the Manfioletti publication cited in the application, which is the first structural characterization of Gas6, uses the A-D domain terminology for the four domains found in Gas6 (from amino- to carboxy-terminus). (A copy of Manfioletti is enclosed.) In addition to structural characterization, numerous functional studies had been undertaken at the time the application was filed, further characterizing Gas6, as well as the domains responsible for its ligand interaction. At page 3, line 29, to page 4, line 4, the WO 2005/007183 publication states:

Apart from a Gla-domain-dependent interaction with phospholipid membranes Gas6 also binds as a ligand to the receptor tyrosine kinases Axl (Ark, Ufo, Tyro7), Sky (Rse, Tyro3, Dtk, Etk, Brt, Tif) and Mer (c-Mer, Eyk, Nyk) by its carboxy-terminal globular G domains. (Citations omitted; Emphasis added.)

This is further corroborated by the section at page 11 of the specification describing

“fragments of Gas6 which lack the A domain as well as fragments which consist essentially of the D domain, such as those described in WO96/28548” (see page 11, lines 11-13). The WO96/28548 publication referred to in the specification again summarizes the knowledge of the structural domains of Gas6 (see WO96/28548, page 6, lines 19-31 and Figs 1 and 2; copy enclosed), recapitulating that the two carboxy-terminal G domains are part of the D domain. WO96/28548, in addition, shows that the carboxy-terminal globular G domains (i.e., the D domain) are in themselves sufficient for receptor binding and activation (see Example 8, page 46 of WO96/28548 and Fig. 7).

All the above information characterizing Gas6 was available well before the earliest priority date of the present application (July 17, 2003). Applicants respectfully submit that a skilled person would recognize which portions of Gas6 were essential to ligand binding. As such, contrary to the Office’s assertion, in view of the knowledge in the art at the time of filing, there is indeed a disclosure of the domain(s) responsible for the ligand activity of Gas6 in the specification as filed that is sufficient for one skilled in the art to recognize that Applicants, at the time of filing, were in possession of the Gas6 mutants and fragments recited in the claims.

Further, the Office cites Examples 7-11 of the revised Written Description Training Materials in support of the written description rejection. Applicants submit that the present case is analogous to Example 11B of the Written Description Training Materials, entitled “Art-Recognized Structure-Function Correlation Present.” Indeed, the current claim language “a Gas6 mutant having at least 95% sequence identity at the amino acid level compared to the wild type Gas6 protein and retaining the function of activating the Gas6 receptors Axl, Mer and Sky” mirrors that of claim 2 of Example 11B, which reads “An isolated nucleic acid that encodes a polypeptide with at least 85% amino acid sequence identity to SEQ ID NO: 2; wherein the polypeptide has activity Y.” If anything, Applicants’ claim language includes more specific limitations than are included in the

exemplary claim. Example 11B of the Written Description Training Materials concludes that the exemplary specification satisfies the written description requirement of 35 U.S.C. 112, first paragraph, with respect to the scope of claim 2, because a correlation exists between structure and function. Applicants submit that this should also apply to the Gas6 mutants encompassed by claim 21 because, as described above, the structure of Gas6 was known at the time of filing and detailed structure-function analysis had been performed.

The Office also asserts that, while the specification provides written description for a physiologically tolerated salt of Gas6, it does not describe the characteristics of physiologically tolerated salts of EPO. Applicants respectfully disagree.

As an initial matter, Applicant note that, throughout the application, physiologically tolerated salts of EPO are described (see, e.g., page 7, lines 5-6, 11, and 32; page 8, lines 6, 19, 23, and 25; page 9, lines 1, 11, and 24; and page 10, lines 8 and 13, of the WO 2005/007183 publication). Thus, one skilled in the art would recognize that physiologically tolerated salts of EPO are encompassed by the specification as filed.

In addition, while the physiologically tolerated salts have indeed been exemplified for Gas6 compounds, they are by no means limited to such compounds. Indeed, the property of being “physiologically tolerated” refers to the salt, not to Gas6 or EPO (which are by definition “physiologically tolerated” as they are normally produced and required in the body). The skilled person would recognize that the description of salts in the specification is not restricted to Gas6, but applies also to EPO. In fact, most of the passage cited by the Office (i.e., from page 11, line 28, to page 12, line 13, of the WO 2005/007183 publication) is a listing of non-toxic, appropriate, salt-forming acids and bases, without mentioning any restriction to Gas6. There is no reason whatsoever why a skilled person would consider these salts as inappropriate for EPO salts, as the “physiologically tolerated” part of description of the salt is by no means linked to the properties of Gas6, but rather relies on the properties of the salt-forming acid or base.

Further, Applicants note that the compounds (e.g., EPO and Gas6) may be administered simultaneously (see, e.g., page 18, lines 12-13, of the WO 2005/007183 publication: "The pharmaceutical compositions and combined preparations according to this invention may be administered orally or in any other suitable fashion"). In this case, EPO and Gas6 salts will typically use the same salt, if only for ease of administration. This basis for the written description rejection may be withdrawn.

New Matter

Claims 21, 30-32 have been rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement, based on the assertion that the phrase "... a point mutant or deletion mutant of erythropoietin which retains stimulation of the production of red blood cells" recited in claim 21, is not supported by the application as filed.

The claims, as amended, recite "erythropoietin with a point mutation." In support of this amendment, Applicants direct the Office's attention to page 12, lines 24-27, of the WO 2005/007183 publication. Here, the specification states:

The expression product of deletion mutants of a synthetic human Epo cDNA, wherein the point mutations and small deletions in helices and interhelical regions of the four alpha helical [sic] bundle motif have been shown to display biological activity. (Emphasis added.)

Applicants submit that the cited passage clearly provides a basis for "deletion mutants of erythropoietin" as well as for "erythropoietin with a point mutation." Applicants also note that the "helices and interhelical regions of the four alpha helical bundle motif" refer to the four alpha helical structure of EPO that was a well-known structural feature of EPO at the time the application was filed (see, e.g., the Boissel publication submitted herewith; Boissel et al.(1993) *J. Biol. Chem.* **268**:15983-15993; Fig. 1A and Fig. 8).

Further, in the above-cited passage, it is stated that these mutants “display biological activity.” For reasons of clarity, Applicants have opted to state, in the claim, what is intended with biological activity of EPO, i.e., stimulation of the production of red blood cells. Support for this amendment can be found, for example, at page 12, lines 14-16, of the WO 2005/007183 publication. Here the specification states:

Erythropoietin or Epo as used herein refers to the naturally occurring human cytokine, produced primarily in the kidney which stimulates the production of red blood cells.

Moreover, Applicants note that the process by which red blood cells (erythrocytes) are formed, erythropoiesis, is exactly the process from which erythropoietin derives its name. For all the above reasons, Applicants submit that the specification provides support for the instant claims, both with the exact wording and in view of the knowledge in the relevant art. Accordingly, this basis of the written description rejection may be withdrawn.

Scope of Enablement

In the Office Action, the Office indicates that the specification is enabling for a method for the treatment of anemia in a patient which comprises administering to the patient a combination of wildtype Gas6 protein (or a physiologically tolerated salt of wildtype Gas6 protein) and wildtype erythropoietin (or Epoetin or Darbepoetin), either simultaneously or sequentially, thereby ensuring a synergistic rescue effect on erythropoiesis in said patient. Applicants do not dispute this characterization.

However, claims 21 and 30-32 are rejected under 35 U.S.C. 112, first paragraph, based on the assertion that the specification fails to enable the full scope of the claims. In particular, the Office asserts that the claims are not limited to specific variants or mutants, and that even if assays were provided to ascertain functionality, this would still “require

an indeterminate quantity of fundamentally unpredictable investigational experimentation of the skilled artisan to generate the infinite number of derivatives encompassed by EPO and Gas6 analogues, mutants, variants or derivatives thereof and screen same for activity." Applicants address these bases for rejection as they apply to the claims as amended.

The claims as amended recite wild type erythropoietin, epoetin, and darbepoetin, which the Office has indicated to be enabled by the specification. In addition, erythropoietin having a modified N-linked glycosylation pattern is recited in the claims. Such EPO variants were known in the art at the time the application was filed (see, for example, US2003/0077753 referred to at page 13, lines 3-4, of the application). Applicants note that US2003/0077753 describes an N-linked glycosylation variant that is biologically active (i.e., stimulates production of red blood cells). In particular, paragraphs [0012] and [0013] of the US2003/0077753 publication, state:

The EPO muteins according to the invention have the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells.

* * *

The invention provides an erythropoietin mutein which has retained the potential N-glycosylation sites at Asn24, Asn38, Asn83, is N-glycosylated at Asn38 and Asn83 but is not N-glycosylated at Asn24.

With regard to the point mutations and deletion mutants of erythropoietin, these have now been limited to erythropoietin with point mutations in the helices or interhelical regions of the four alpha helical bundle motif, where the mutant stimulates the production of red blood cells and deletion mutants of erythropoietin in the helices or interhelical regions of the four alpha helical bundle motif, where the mutant stimulates the production of red blood cells. Such point mutants and deletion mutants have been shown to display biological activity (see, e.g., Bittorf *et al.* (1993) *FEBS Lett* **336**, 133-136 and Boissel *et al.* (1993) *J. Biol. Chem.* **268**, 15983-15993; cited at page 12, lines 24-28 of the

specification; copies of these references are submitted herewith). Point and deletion mutants encompassed by the claims, as well as their properties, are discussed throughout the Bittorf and Boissel references. In particular, the Office's attention is directed to Figs. 3B, 3C, and 8 of the Boissel reference. These figures list different mutants and their specific activity. In addition, Tables I and II of the Bittorf reference, respectively, list mutants and show their specific activity. Thus, for such mutants, no further testing is required with regard to generating and screening the molecules for activity.

Turning to Gas6, as explained above, the structural information of Gas6 was readily available to the skilled person at the time of filing the application. In particular, Applicants again direct the Office's attention to the passage on Gas6 structure and function in the specification (page 3, line 14, to page 4, line 12, of the WO 2005/007183 publication), the references cited therein, and to WO96/28548, which is cited in the application at page 11, lines 7-13, of the WO 2005/007183 publication.

As already outlined above in reply to the written description rejection, the specification teaches that the carboxy-terminal globular G domains of Gas6 are responsible for receptor binding. This is further validated by reference to WO96/28548 (entitled "Receptor activation by Gas6"), where different deletion mutants again show that the D domain (containing the 2 carboxy-terminal G domains) is essential for binding and that the A domain can be deleted without losing functional activity.

The Office states (page 8):

The specification fails to teach the A or D domain of Gas6. The specification fails to teach (i) which portion of Gas6 protein is critical to and (ii) what modifications (e.g., substitutions, deletions or additions) one can make to Gas6 [that] will result in protein mutants with the same functions as the Gas6 wildtype protein.

Applicants strongly disagree.

As an initial matter, Applicants disagree with the assertion that the specification

fails to teach which portion of Gas6 is critical, as the structural important parts of Gas6 are indeed discussed at pages 3 and 4 of the application. As stated in section 2164.01 of the M.P.E.P., a patent need not teach, and preferably omits, what is well known in the art. The M.P.E.P. cites *In re Buchner*, 929 F.2d 660, 661, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984). As the domain structure, including the A-D domains, of Gas6 was well known (e.g., Manfioletti *et al.* (1993), cited in the application), and as it was known which domains were important for binding and functional activity of Gas6 (e.g., WO96/28548, cited in the application, particularly Example 8 and Figure 7), Applicants submit that the skilled person knew which portions of Gas6 were functionally and structurally important for Gas6 function at the time of filing the application.

At page 11, lines 7-13, of the WO 2005/007183 publication, the specification states:

As used herein "variants" refers to modified Gas6 proteins with a length differing from wild type Gas6 obtained by removal or addition of one or more amino acids (either N-terminal, C-terminal or internally, for instance the variants which are less or not γ -carboxylated, fragments of Gas6 which lack the A domain as well as fragments which consist essentially of the D domain, such as those described in WO96/28548.

Clearly, variants of Gas6 were known in the art years before the filing date of the present application. As a result, the mutations or deletions in Gas6 need not be "randomly selected" as asserted by the Office, and obtaining a mutant with the activity required by the claims does not involve undue experimentation. As the domains necessary for binding and functional activity were already well delineated in the art at the time of filing, Applicants submit that the skilled person could readily identify fragments of Gas6 lacking

the A domain, fragments of Gas6 consisting essentially of the D domain, as well as Gas6 mutants having at least 95% sequence identity at the amino acid level compared to the wild type Gas6 protein and retaining the function of activating the Gas6 receptors Axl, Mer, and Sky. Moreover, as is evident from the references cited in the specification, deletion mutants that retain their function were known in the art at the time of filing. For all the above reasons, Applicants submit that making and using the claimed invention within the full scope of the claims as amended cannot constitute undue experimentation. The scope of enablement rejection may be withdrawn.

Rejection under 35 U.S.C. §112, Second Paragraph

Claims 21 and 30-32 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. In particular, claim 21 is asserted to recite an improper Markush group for reciting both “or” and “and.” Claim 21 as amended only recites “and” in the Markush group, rendering this objection moot.

Also, claim 21 was asserted to be indefinite because of the repetition of “glycosylation variant, point mutant or deletion mutant” of EPO. As noted above, the Markush groups as amended only recite “and.” Applicants submit that the claim as amended clarifies that recitation of “said” applies to each member of the group.

Finally, claim 21 was asserted to be unclear because of the recitation “a Gas6 mutant having at least 95% sequence identity at the amino acid level [of] said Gas6 fragment.” This term has been deleted from the claims and has been replaced with “compared to the wild type Gas6 protein.” Applicants submit that the wild type Gas6 sequence was known in the art at the time of filing and need not be recited in the claim. The specification, at page 10, lines 17-20, of the WO 2005/007183 publication states:

The reference to Gas6, a mutant, variant or derivative thereof (generally referred to herein as Gas6 compounds) includes the Gas6 protein as encoded by the human Gas6 gene (Manfioletti *et al.* (1993) Molec. Cell

Biol 13, 4976-4985).

Applicants submit that the specification of a comparison to the wild type Gas6 sequence in the present claims is not indefinite based on the long-standing characterization of the wild type Gas6 sequence.

Claim objections

Claim 21 was objected to because “erythropoietin” was misspelled. The spelling of “erythropoietin” has been corrected in the claims as amended. This basis for objection may be withdrawn.

CONCLUSION

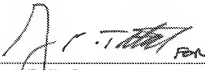
Applicants submit that the application is now in condition for allowance, and such action is hereby respectfully requested.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/12, 15/63, 15/85, C07K 14/745, A61K 38/36	A1	(11) International Publication Number: WO 96/28548
		(43) International Publication Date: 19 September 1996 (19.09.96)

(21) International Application Number: PCT/US96/03031

(22) International Filing Date: 5 March 1996 (05.03.96)

(30) Priority Data:
08/402,233 10 March 1995 (10.03.95) US
08/438,861 10 May 1995 (10.05.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/438,861 (CIP)
Filed on 10 May 1995 (10.05.95)

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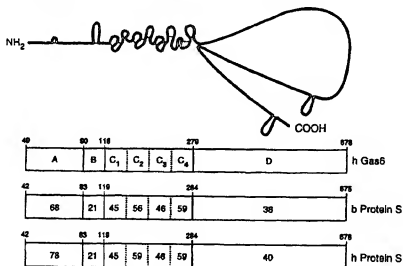
(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published*With international search report.*

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: RECEPTOR ACTIVATION BY GAS6

Gla Region Loop EGF-like Repeats SHBG-like Domain



(57) Abstract

An activator of the Rse and Mer receptor protein tyrosine kinases has been identified. The activator is encoded by growth arrest-specific gene 6 (gas6). Accordingly, the invention provides a method of activating the Rse or Mer receptor using gas6 polypeptide. Furthermore, the invention provides a method of enhancing survival, proliferation or differentiation of cells having the Rse or Mer receptor incorporated in their cell membranes which involves those cells to gas6 polypeptide. The types of cells which can be treated according to the method include glial cells such as Schwann cells and mononuclear cells. Kits and articles of manufacture which include gas6 polypeptide are further provided. The invention also relates to gas6 variants.

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RECEPTOR ACTIVATION BY GAS6

BACKGROUND OF THE INVENTION

Field of the Invention

- The invention relates generally to methods of activating the Rse or Mer tyrosine kinase receptors. More particularly, the invention relates to methods of enhancing survival, proliferation and/or differentiation of cells comprising the Rse receptor (such as glial cells) or Mer receptor (e.g., monocytes) using gas6. The invention also relates to gas6 variants, particularly those which are less γ -carboxylated than gas6 isolated from nature.

Description of Related Art

- Specific signals that control the growth and differentiation of cells in developing and adult tissues often exert their effects by binding to and activating cell surface receptors containing an intrinsic tyrosine kinase activity. Mark *et al.* recently described the human and murine complementary DNA sequences of the receptor tyrosine kinase Rse that is preferentially expressed in the adult brain (Mark *et al.*, *J. Biol. Chem.*, 269:10720 [1994]). The extracellular domain of Rse receptor is composed of two immunoglobulin-like (Ig-L) repeats followed by two fibronectin type III repeats. Complementary DNA sequences encoding proteins identical to human (Ohashi *et al.*, *Oncogene* 9:699 [1994]) and murine Rse (Lai *et al.*, *Oncogene* 9:2567 [1994]) have been reported independently, and termed Sky and Tyro3, respectively. See also Fujimimoto and Yamamoto *Oncogene* 9: 693 (1994) concerning the murine equivalent to Rse they call brr and Dai *et al.* *Oncogene* 9: 975 (1994) with respect to the human molecule they call tif.

- The expression of Rse in various tissues has been investigated. Lai *et al.*, *supra*, found that, in the adult brain, Rse mRNA is localized in neurons of the neocortex, cerebellum and hippocampus. Schulz *et al.* similarly found that Rse is expressed at high levels in the cerebral cortex, the lateral septum, the hippocampus, the olfactory bulb and in the cerebellum. The highest levels of Rse expression in the brain were found to be associated with neurons. (Schulz *et al.* *Molec. Brain Res.* 28: 273-280 [1995]). In the central nervous system (CNS) of mice, the expression of Rse is detected at highest levels during late embryonic stages and post birth, coincident with the establishment and maintenance of synaptic circuitry in cortical and hippocampal neurons (Lai *et al.*, *supra* and Schneider *et al.*, *Cell* 54:787-793 [1988]). This process is believed to be regulated locally, by cells that are in direct contact or positioned close to one another. By Northern blot analysis, Mark *et al.*, *supra*, found that high levels of Rse mRNA were present in samples of RNA from the brain and kidney. Dai *et al.*, *supra* found that Rse was highly expressed in human ovary and testes. The expression of Rse in various human cell lines was also analyzed by Mark *et al.*, *supra*. Little, or no, Rse mRNA was detected by Northern blotting of mRNA samples from the monocyte cell line THP-1 or the lymphoblast-like RAJI cells. However, the Rse transcript was detected in a number of hematopoietic cell lines, including cells of the myeloid (i.e., myelogenous leukemia line K562 and myelomonocytic U937 cells) and the megakaryocytic leukemia lines DAMI and CMK11-5, as well as the human breast carcinoma cell line MCF-7. In the cell lines examined, the highest level of expression was observed in Hep 3B cells, a human hepatocarcinoma cell line.

Rse is structurally related to Axl (also known as Ufo or Ark) and shares 43% overall amino acid sequence identity with this tyrosine kinase receptor. See O'Bryan *et al.*, *Mol. Cell. Biol.* 11:5016 (1991),

Janssen *et al.*, *Oncogene* 6:2113 (1991), Rescigno *et al.*, *Oncogene* 5:1908 (1991) and Bellosta *et al.* 15: 614 (1995) concerning Axl. Rse and Axl, together with Mer (Graham *et al.*, *Cell Growth Differ.* 5:647 [1994]), define a class of receptor tyrosine kinases whose extracellular domains resemble neural cell recognition and adhesion molecules (reviewed by Ruitishauser, U. in *Current Opin. Neurobiology* 3:709 [1993] and Brummendorf and Rathjen in *J. Neurochemistry* 61:1207 [1993]). Like Rse, Axl is also expressed in the nervous system, but is more widely expressed than Rse in peripheral tissues.

Mer mRNA is detected in peripheral blood mononuclear cells, bone marrow mononuclear cells and in monocytes, but not in granulocytes. Despite the fact that Mer mRNA is expressed in neoplastic B and T cell lines, it is not detected in normal B or T lymphocytes (Graham *et al.*, *Cell Growth Differ.* 5:647 [1994]). Mer is widely expressed in human tissues, but the highest levels of Mer mRNA are detected in the testis, ovary, prostate, lung and kidney (Graham *et al.*, *Cell Growth Differ.* 5:647 [1994]).

Disregulated expression of Mer, Rse and Axl is associated with cellular transformation. For example, Axl was isolated from DNA of patients with chronic myelogenous leukemia (O'Bryan *et al.*, *supra*) and chronic myeloproliferative disorder (Janssen *et al.*, *supra*) using a transfection/tumorigenicity assay. Mer was cloned from a neoplastic B cell line and is expressed in numerous transformed T acute lymphocytic leukemia cell lines (Graham *et al.*, *supra*). Rse and Axl, when overexpressed in fibroblasts, induce cellular transformation (O'Bryan *et al.*, *supra*; Ohashi *et al.*, *Oncogene* 9:669 [1994]; Taylor *et al.*, *J. Biol. Chem.* 270:6872-6880 [1995]; and McCloskey *et al.*, *Cell Growth and Diff.* 5:1105-1117 [1994]). Rse mRNA and protein are also overexpressed in mammary tumors derived from transgenic animals that overexpress either the *wnt-1* or *lgf-3* oncogenes (Taylor *et al.*, *J. Biol. Chem.* 270: 6872-6880 [1994]).

Putative ligands for the Rse and Axl receptors have been reported. Varnum *et al.* *Nature* 373:623 (1995) and Stitt *et al.* *Cell* 80: 661-670 (1995) recently reported that gas6 (for growth arrest-specific gene 6) is a ligand for Axl. Gas6 belongs to a set of murine genes which are highly expressed during serum starvation in NIH 3T3 cells (Schneider *et al.*, *Cell* 54:787-793 [1988]). These genes were designated growth arrest-specific genes, since their expression is negatively regulated during growth induction. The human homolog of murine gas6 was also cloned and sequenced by Manfioletti *et al.* in *Molec. Cell Biol.* 13(8):4976-4985 (1993). They concluded that gas6 is a vitamin K-dependent protein and speculated that it may play a role in the regulation of a protease cascade relevant in growth regulation. Gas6 is expressed in a variety of tissues including the brain. See also Colombo *et al.* *Genome* 2:130-134 (1992) and Ferrero *et al.* *J. Cellular Physiol.* 158:263-269 (1994) concerning gas6.

Stitt *et al.*, *supra* further reported that protein S is the ligand for Tyro3. Protein S is a vitamin K-dependent plasma protein that functions as an anticoagulant by acting as a cofactor to stimulate the proteolytic inactivation of factors Va and VIIIa by activated protein C. Reviewed in Easmon *et al.* *Atheroscler. Thromb.* 12:135 (1992). Accordingly, protein S is an important negative regulator of the blood-clotting cascade. See Walker *et al.*, *J. Biol. Chem.* 255:5521-5524 (1980), Walker *et al.*, *J. Biol. Chem.* 256:11128-11131 (1981), Walker *et al.*, *Arch. Biochem. Biophys.* 252: 322-328 (1991), Griffin *et al.* *Blood* 79: 3203 (1992) and Easmon, D., *Atheroscler. Thromb.* 12:135 (1992). The discovery that about half of the protein S in human plasma is bound to C4BP further supports the notion that protein S is involved in the complement cascade. Dahlback *et*

al., PNAS(USA) 78: 2512-2516 (1981). The role of protein S as a mitogen for smooth muscle cells has also been reported. Gasic *et al.*, PNAS (USA) 89:2317-2320 (1992).

Protein S can be divided into four domains (see Figs. 1A, 1C and 1D herein). Residues 1-52 (Region A) are rich in γ -carboxyglutamic acid (Gla) residues which mediate the Ca^{2+} dependent binding of protein S to negatively charged phospholipids (Walker, J. Biol. Chem. 259:10335 [1984]). Region B includes a thrombin-sensitive loop. Region C contains four epidermal growth factor (EGF)-like repeats. Region D is homologous to the steroid hormone binding globulin (SHBG) protein (Hammond *et al.*, FEBS Lett. 215:100 [1987]). As discussed by Joseph and Baker (FASEB J. 6:2477 [1994]), this region is homologous to domains in the A chain of laminin (23% identity) and merosin (22% identity) and to a domain in the *Drosophila* crumbs (19%).

Murine and human gas6 cDNAs encode proteins having 43 and 44% amino acid sequence identity respectively to human protein S.

SUMMARY OF THE INVENTION

The foregoing invention relates to gas6 variants which are essentially not γ carboxylated or are substantially less γ carboxylated than gas6 derived from an endogenous source of the molecule. Examples of such variants include gas6 variants lacking one or more glutamic acid residues from the A domain of gas6 which are normally γ carboxylated, fragments of gas6 which lack the A domain as well as fragments which consist essentially of the D domain of gas6 (or a G domain fragment of gas6).

The invention further provides a method of activating Rse or Mer receptor by exposing a cell (preferably a human cell) comprising the Rse or Mer receptor to exogenous gas6 in an amount effective to activate the Rse or Mer receptor. The Rse or Mer receptor is normally cell-bound and the gas6 is preferably human gas6. The invention also provides a method of enhancing survival, proliferation and/or differentiation of a cell which has the Rse or Mer receptor incorporated in the cell membrane thereof by exposing the cell to gas6 in an amount effective to enhance survival, proliferation and/or differentiation of the cell. The cell may be a neuron or a glial cell, such as a Schwann cell, or a monocyte (e.g. a macrophage). The cell may be present in cell culture or in a mammal (e.g. a human) which is suffering from a neurologic disease or disorder. Often, the gas6 is provided in a physiologically acceptable carrier.

The invention also provides kits and articles of manufacture comprising gas6 polypeptide. The article of manufacture usually comprises instructions for using the gas6 in an *in vitro* cell culture or for administering the gas6 to a mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1D provide a schematic representation of the structure of protein S and gas6 (Fig. 1A) and comparison of the amino acid homology between the bovine (b) and human (h) forms of protein S (Figs. 1C and 1D, respectively) with human gas6 (Fig. 1B). For h gas6, boxes represent the Gla region (i.e. the A domain), the loop region (i.e. the B domain), the 4 EGF-like repeats (labeled C₁-C₄) which form the C domain, and the region homologous to sex-hormone binding globulin (i.e. the D domain), which is also related to the G domains of laminin A chain and merosin and to *Drosophila* crumbs protein. The percentage of amino acid identity shared

between h gas6 and either b protein S or h protein S is indicated within the corresponding boxes. The amino acids at the boundaries of each of the regions are indicated above the boxes.

Fig. 2 shows a comparison of the amino acid sequences of murine gas6 (m gas6) [SEQ ID NO:1], h gas6 [SEQ ID NO:2] and h protein S [SEQ ID NO:3]. Residues of the "pre" and "pro" sequences are indicated (with the arrow indicating the last residue of each precursor sequence). The A-D domains are delineated, as are the two G domains which reside in the D domain (i.e. G domain 1 and G domain 2).

Figs. 3A-3D are graphs depicting characterization of the Rse-L in fetal bovine serum (FBS). Fig. 3A shows binding of 125 I-Rse-IgG as a function of FBS concentration. Binding, percent of total counts added that are membrane associated ($100 \times \text{B/T}$, i.e. bound/total), is plotted as a function of FBS concentration. The data were fit to a 4 parameter model which gave an EC_{50} of 0.58% v/v. Fig 3B illustrates binding of 125 I-Rse-IgG as a function of Ca^{2+} concentration, with constant FBS concentration. Binding was performed as in Fig. 3A either in the presence of 10% dialyzed FBS or in its absence and varying the concentration of added Ca^{2+} . The EC_{50} of Ca^{2+} as judged by a 4 parameter fit to the data is 0.18 mM. Fig 3C is a Scatchard analysis of 125 I-Rse-IgG binding to CMK11-5 membranes mediated by FBS. A single concentration of 125 I-Rse-IgG, FBS and Ca^{2+} was used with increasing concentrations of unlabeled Rse-IgG, and binding plotted vs the ratio of bound and free (B/F) after correction for nonspecific binding. Experiments at both 1% ($K_d = 0.82$ nM) and 10% ($K_d = 2.2$ nM) FBS are shown. Fig 3D is a KIRA analysis of dose dependent activation of Rse phosphorylation by the Q-sepharose enriched (QSE) fraction of FBS. The inset shows Rse-L activity was specifically neutralized by incubation with Rse-IgG. Rse phosphorylation is shown in serum starved cells (-); or cells treated with QSE fraction in the absence of added IgG proteins (QSE); or with QSE incubated with Rse-IgG or CD4-IgG as indicated.

Fig. 4 is a flow chart/cartoon illustrating the KIRA ELISA for the Rse receptor described in Example 4.

Fig. 5 displays inhibition of binding of 125 I-Rse-IgG to gas6 by unlabeled Rse-IgG. Increasing amounts of unlabeled Rse-IgG were added to tubes with constant 125 I-Rse-IgG and gas6. A nonlinear least squares fit to the data using a single class of sites gave an estimated equilibrium dissociation constant of 0.46 ± 0.04 nM. The inset shows a Scatchard plot of bound (B) vs bound/free (B/F) after correction for nonspecific binding.

Figs. 6A-6C show Rse-L activity in astrocyte cultures. To determine if astrocytes secrete Rse ligand, serum free media that was conditioned for 3 days was concentrated 10-fold in a Centricon-10 centrifugal ultrafiltration device, and added directly to assay tubes to give the final concentrations indicated. In Fig. 6A binding of 125 I-Rse-IgG to CMK11-5 membranes was enabled by addition of astrocyte conditioned medium (ACM), with a half maximum effect achieved at 13% v/v ACM. Fig. 6B is a KIRA analysis of phosphorylation of Rse by ACM. Fig. 6C shows that the phosphorylation of Rse by ACM was inhibited by incubation with Rse-IgG, but not CD4-IgG. Neutralization was carried out as described in Fig. 3 legend.

As shown in Fig. 7, a deletion analysis of gas6 indicated that the G domains are sufficient for binding to Rse *in vitro*. Epitope tagged (gD) gas6 or protein S, or N-terminal truncation variants of gas6 (containing the indicated residues) were constructed and transiently expressed in 293 cells essentially following the procedure described in Example 6. Proteins of the correct molecular weight could be detected in unfractionated (input) cell supernatants using an antibody directed against the epitope tag. In contrast to protein S, the gas6 derivatives

were precipitated from the cell supernatants by Rse-IgG. The binding was specific to the extracellular domain of Rse because proteins were not precipitated by control human Fc. For quantification purposes, the unfractionated (input) lanes represented 20% of the material used for precipitation.

Fig. 8 shows that gas6 induces the proliferation of P45 rat Schwann cells in a dose responsive fashion.

- 5 Cells were plated in 24-well plates in F12/DME medium with 10 μ g/ml insulin and transferrin and 5 μ g/ml Vitamin E with the indicated concentrations of recombinant human gas6. Cells were counted with a Coulter counter after 48 h. The mean and standard deviation for six wells for each treatment are shown.

- Fig. 9 illustrates that gas6 induced proliferation of p45 rat Schwann cells is neutralized by Rse-IgG. Cells were plated as described in Fig. 8 legend. Control cells received no further additions. All other cells were
10 treated with two different purifications of gas6 (i.e. lot #15 and lot #9) and 10 μ g/ml of either Rse-IgG (labeled Rse) or CD4-IgG (labeled CD4Fc).

Fig. 10 shows a dose response curve for activation of Rse phosphorylation in the KIRA assay as described in Example 10.

- Fig. 11 illustrates ion exchange chromatography of media conditioned by cells expressing human recombinant gas6. Media (700 ml) was dialyzed against buffer A (50 mM Tris-HCl pH 7.5, 5 mM benzamidine), adjusted to 0.1 % CHAPS, and loaded on a 6 ml Resource-Q column (Pharmacia) at 10 ml/minute. The column was washed with buffer A, and eluted with a 70 ml linear gradient of 0 to 0.4 M NaCl in buffer A at a flow rate of 1.0 ml/min collecting fractions of 2.0 ml. The fractions were analyzed for their ability to bind and activate Rse using the barium chloride binding method described in Example 6 and the KIRA assay described in Example 4. The binding activity is expressed as the percent of total radioactivity added which
20 is precipitated by barium chloride. The KIRA activity is expressed in units/ml relative to a standard.

- Figs. 12A-12C depict the effect of gas6 and other growth factors on human Schwann cell growth and DNA synthesis. All data presented as mean \pm standard error (n=4). Fig. 12A shows dose responsive curves of human Schwann cells to gas6 in different conditions. Cell numbers were counted with coulter counter at 84 hours
25 after culture with the indicated concentrations of gas6. Fig. 12B shows that gas6 increased thymidine incorporation in Schwann cells cultured as in Fig. 12A in the presence of different concentrations of gas6. ³H-[methyl]-thymidine (0.5 μ Ci/ml) was added at 48 hours of culture. Cells were harvested at 96 hours of culture and processed for measurement of the radioactivity incorporated in DNA. Fig. 12C shows the influence of growth factors on Schwann cell growth in the presence of 8F. Schwann cells were plated in 8F with or without
30 PDGF (10 ng/ml, basic FGF (20 ng/ml), IL-1 α (1 ng/ml), TGF- β 1 (1 ng/ml) and gas6 (30 ng/ml). Cell numbers were counted after 108 hours.

- Fig. 13 illustrates a time course of human Schwann cell growth in culture. Human Schwann cells were plated at 2×10^4 cells/well in 24 well multiplates in F12/DME (1:1) supplemented with 8F with or without gas6 or 10% dia-filtered fetal bovine serum (FBS). Four wells of cultures were taken from each group for cell
35 counting every 24 hours. Data shown are mean \pm standard error (n=4).

Fig. 14 depicts neutralization of gas6 induced phosphorylation of Rse by receptor-Fc fusion proteins measured in a KIRA assay. The percentage of Rse phosphorylation observed in CHO Rse.gD cells treated with purified gas6 in the presence of the indicated concentrations of receptor fusion protein relative to that observed in cells treated with just gas6 is shown.

Figs. 15A-15D show a kinetic analysis of ligand binding to Mer-Fc. Mer-Fc was coupled to the carboxymethylated dextran layer on the surface a BIAcore™ biosensor. Purified gas6 (Figs. 15A, B and C) or Protein S (Fig. 15D) at a concentration of either 100 nM (broken line) or 140 nM (solid line) was injected over the surface of the biosensor at 160 sec. At 340 seconds, the injector loop was switched to buffer to follow dissociation. The binding of gas6 to Mer-Fc on the chip was blocked by preincubation with soluble Mer-Fc (Fig. 15B) but not CD4-Fc (Fig. 15C). No binding of Protein S to Mer-Fc was observed (Fig. 15D).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

1. Definitions

As used herein, the terms "gas6" and "gas6 polypeptide" (unless indicated otherwise) refer to a polypeptide which is able to activate the Rse receptor or Mer receptor and encompass the mature, pre-, prepro- and pro- forms of gas6 polypeptide, either purified from a natural source, chemically synthesized or recombinantly produced. The present definition specifically includes "human" gas6 polypeptide comprising the amino acid sequence published in Manfioletti *et al.*, *Mol. Cell. Biol.* 13(8):4976-4985 (1993) (available from EMBL/GenBank/DBJ under accession number X59846) and other mammalian gas6 polypeptides (such as murine gas6, see Manfioletti *et al.*, *supra*). Where the gas6 polypeptide has the amino acid sequence of a gas6 polypeptide found in nature, it is referred to herein as a "native" or "native sequence" polypeptide regardless of the method by which it is produced (e.g. it can be isolated from an endogenous source of the molecule or produced by synthetic techniques).

Gas6 has various amino acid "regions" or "domains" which are delineated in Figs. 1A-B and Fig. 2. The "A domain" or "Gla region" at the amino terminus of the polypeptide has residues which are rich in γ -carboxyglutamic acid (Gla residues) which appear to mediate calcium dependent binding of gas6 to negatively charged phospholipids in cell membranes. The A-domain stretches from about residue 46-86 of murine gas6 and about residue 49-89 of human gas6. The following "B domain" comprises a thrombin sensitive "loop" and extends from about residue 87-114 of murine gas6 and about residue 90-117 of human gas6. The third domain called the "C domain" herein has four epidermal growth factor (EGF)-like repeats (C₁-C₄ in Fig. 1B). This C domain extends from about residue 115-275 of murine gas6 and about residue 118-278 of human gas6. The remaining "D domain" is homologous to steroid hormone binding globulin (SHBG) protein and comprises about residues 276-673 of murine gas6 and residues 279-678 of human gas6. The D domain comprises a pair of "G domains" called "G Domain 1" (i.e. about residues 311-468 for murine gas6 and about residues 314-471 for human gas6) and "G Domain 2" (i.e. about residues 500-666 for murine gas6 and about residues 503-671 for human gas6).

The terms "gas6" and "gas6 polypeptide" also encompass "variants" or "mutants" of native gas6. Such variants include fragments of the human gas6 sequence; polypeptides wherein one or more amino acid residues are added at the N- or C-terminus of, or within, the human gas6 sequence; one or more amino acid residues are deleted, and optionally substituted by one or more amino acid residues; and derivatives of the above proteins, polypeptides, or fragments thereof, wherein an amino acid residue has been covalently modified so that the resulting product is a non-naturally occurring amino acid. Gas6 variants may be made synthetically, for example, by site-directed or PCR mutagenesis, or may exist naturally, as in the case of allelic forms and other naturally

occurring variants of the translated amino acid sequence set forth in Manfioletti *et al.* that may occur in human and other animal species.

A gas6 variant is included within the scope of the invention provided that it is functionally active. As used herein, "functionally active" and "functional activity" in reference to gas6 means that the gas6 is able to activate the Rse receptor and/or Mer receptor and/or promote the proliferation, survival, and/or differentiation of cells comprising the Rse receptor or Mer receptor such as neurons, glial cells or monocytic cells. A "glial cell" is derived from the central and peripheral nervous system and can be selected from oligodendroglial, astrocyte, ependymal, or microglial cells as well as satellite cells of ganglia and the neurolemmal or Schwann cells around peripheral nerve fibers. A "monocytic cell" is a mononuclear leukocyte such as a macrophage.

Often gas6 variants will share at least about 75% (preferably greater than 80% and more preferably greater than 90%) sequence identity with the translated amino acid sequence encoding mature gas6 or fragments thereof after aligning the sequences to provide for maximum homology, as determined, for example, by the Fitch *et al.*, *PNAS(USA)* 80:1382-1386 (1983), version of the algorithm described by Needleman *et al.*, *J. Mol. Biol.* 48:443-453 (1970). In order to screen for functionally active gas6 variants, a variant can be subjected to one or more of the following functional activity tests/assays:

- (a) Receptor activation assays which measure downregulation or activation of receptor tyrosine kinase activity (e.g. western blotting using an anti-phosphotyrosine antibody to determine whether the variant is able to activate Rse receptor or Mer receptor, see Example 3 herein).
- (b) KIRA ELISA to determine Rse or Mer receptor activation-capability of the variant as described in Example 4 below.
- (c) Schwann cell proliferation assay to establish whether or not the variant is able to enhance Schwann cell proliferation in cell culture. See Example 9 herein.

Amino acid sequence variants of gas6 can be prepared by introducing appropriate nucleotide changes into gas6 DNA and thereafter expressing the resulting modified DNA in a host cell, or by *in vitro* synthesis. Such variants include, for example, deletions from, or insertions or substitutions of, amino acid residues within the gas6 amino acid sequence set forth in Manfioletti *et al.* Any combination of deletion, insertion, and substitution may be made to arrive at an amino acid sequence variant of gas6, provided that such variant possesses the desired characteristics described herein. Changes that are made in the amino acid sequence to arrive at an amino acid sequence variant of gas6 may also result in further modifications of gas6 upon its expression in host cells, for example, by virtue of such changes introducing or moving sites of glycosylation.

There are two principal variables in the construction of amino acid sequence variants of gas6: the location of the mutation site and the nature of the mutation. These are variants from the human gas6 amino acid sequence, and may represent naturally occurring allelic forms of gas6, or predetermined mutant forms of gas6 made by mutating gas6 DNA, either to arrive at an allele or a variant not found in nature. In general, the location and nature of the mutation chosen will depend upon the gas6 characteristic to be modified.

For example, due to the degeneracy of nucleotide coding sequences, mutations can be made in the human gas6 nucleotide sequence without affecting the amino acid sequence of the gas6 encoded thereby. Other mutations can be made that will result in a gas6 that has an amino acid sequence different from that set forth in Manfioletti *et al.*, but which is functionally active. Such functionally active amino acid sequence variants of gas6

are selected, for example, by substituting one or more amino acid residues in the human gas6 amino acid sequence with other amino acid residues of a similar or different polarity or charge.

One useful approach is called "alanine scanning mutagenesis". Here, an amino acid residue or group of target residues is/are identified (e.g., charged residues such as arg, asp, his, lys, and glu) and, by means of recombinant DNA technology, replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine) to affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Cunningham *et al.*, *Science* 244:1081-1085 (1989). Those domains demonstrating functional sensitivity to the substitutions are then refined by introducing further or other variants at or for the sites of substitution.

Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation *per se* need not be predetermined. For example, to optimize the performance of a mutation at a given site, ala scanning or random mutagenesis is conducted at the target codon or region and the expressed gas6 variants are screened for functional activity as discussed above.

Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably about 1 to 10 residues, and typically are contiguous. Deletions from regions of substantial homology with other tyrosine kinase receptor ligands, for example, are more likely to affect the functional activity of gas6. Generally, the number of consecutive deletions will be selected so as to preserve the tertiary structure of gas6 in the affected domain, e.g., β -pleated sheet or α helix. Preferred deletion mutants include those which lack one or more glutamic acid residues in the A domain of gas6 (i.e. those E residues in the A domain of gas6 shown in Fig. 2) or lack the A domain entirely. A preferred deletion mutant of gas6 is the D domain of gas6 or one of the G domains thereof.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions ranging in length from one amino acid residue to polypeptides containing a hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions (i.e., insertions made within the human gas6 amino acid sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5, most preferably 1 to 3. Examples of terminal insertions include gas6 with an N-terminal methionyl residue (such as may result from the direct expression of gas6 in recombinant cell culture), and gas6 with a heterologous N-terminal signal sequence to improve the secretion of gas6 from recombinant host cells. Other insertions include the fusion to the N- or C-terminus of gas6 of immunogenic polypeptides (for example, bacterial polypeptides such as β -lactamase or an enzyme encoded by the *E. coli* trp locus, or yeast protein), and C-terminal fusions with proteins having a long half-life such as immunoglobulin constant regions, albumin, or ferritin, as described in PCT Pub. No. WO 89/02922 (published April 6, 1989).

The third group of variants are those in which at least one amino acid residue in the gas6 amino acid sequence, and preferably only one, has been removed and a different residue inserted in its place. The sites of greatest interest for making such substitutions are in the regions of the gas6 amino acid sequence that have the greatest homology with other tyrosine kinase receptor ligands. Those sites are likely to be important to the functional activity of the gas6. Accordingly, to retain functional activity, those sites, especially those falling within a sequence of at least three other identically conserved sites, are substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 1 under the heading of "Preferred Substitution".

If such substitutions do not result in a change in functional activity, then more substantial changes, denominated "Exemplary Substitutions" in Table 1, or as further described below in reference to amino acid classes, may be introduced and the resulting variant gas6 analyzed for functional activity.

TABLE 1

	Original Residue	Exemplary Substitutions	Preferred Substitution
5	Ala (A)	val; leu; ile	val
	Arg (R)	lys; gln; asn	lys
	Asn (N)	gln; his; lys; arg	gln
	Asp (D)	glu	glu
10	Cys (C)	ser	ser
	Gln (Q)	asn	asn
	Glu (E)	asp	asp
	Gly (G)	ala; pro	ala
	His (H)	asn; gln; lys; arg	arg
15	Ile (I)	leu; val; met; ala; phe; norleucine	leu
	Leu (L)	norleucine; ile; val; met; ala; phe	ile
	Lys (K)	arg; gln; asn	arg
	Met (M)	leu; phe; ile	leu
	Phe (F)	leu; val; ile; ala; tyr	leu
20	Pro (P)	ala	ala
	Ser (S)	thr	thr
	Thr (T)	ser	ser
	Trp (W)	tyr; phe	tyr
	Tyr (Y)	trp; phe; thr; ser	phe
25	Val (V)	ile; leu; met; phe; ala; norleucine	leu

Insertional, deletional, and substitutional changes in the gas6 amino acid sequence may be made to improve the stability of gas6. For example, trypsin or other protease cleavage sites are identified by inspection of the encoded amino acid sequence for an arginyl or lysinyl residue. These are rendered inactive to protease by substituting the residue with another residue, preferably a basic residue such as glutamine or a hydrophobic residue such as serine; by deleting the residue; or by inserting a prolyl residue immediately after the residue. Also, any cysteine residues not involved in maintaining the proper conformation of gas6 for functional activity may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant crosslinking.

DNA encoding amino acid sequence variants of gas6 is prepared by a variety of methods known in the art. These methods include, but are not limited to, isolation from a natural source (in the case of naturally

occurring amino acid sequence variants of gas6) or preparation by site-directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared DNA encoding a variant or a non-variant form of gas6.

Site-directed mutagenesis is a preferred method for preparing substitution, deletion, and insertion variants of gas6 DNA. This technique is well known in the art (see, e.g., Zoller *et al.*, Meth. Enz. 100:4668-500 [1983]; Zoller *et al.*, Meth. Enz. 154:329-350 [1987]; Carter, Meth. Enz. 154:382-403 [1987]; and Horwitz *et al.*, Meth. Enz. 185:599-611 [1990]), and has been used, for example, to produce amino acid sequence variants of trypsin and T4 lysozyme, which variants have certain desired functional properties. Perry *et al.*, Science 226:555-557 (1984); and Craik *et al.*, Science 228:291-297 (1985).

Briefly, in carrying out site-directed mutagenesis of gas6 DNA, the gas6 DNA is altered by first hybridizing an oligonucleotide encoding the desired mutation to a single strand of such gas6 DNA. After hybridization, a DNA polymerase is used to synthesize an entire second strand, using the hybridized oligonucleotide as a primer, and using the single strand of gas6 DNA as a template. Thus, the oligonucleotide encoding the desired mutation is incorporated in the resulting double-stranded DNA.

PCR mutagenesis is also suitable for making amino acid sequence variants of gas6. See Higuchi, in PCR Protocols, pp.177-183 (Academic Press, 1990); and Vallette *et al.*, Nuc. Acids Res. 17:723-733 (1989). Briefly, when small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.*, Gene 34:315-323 (1985). The starting material is the plasmid (or other vector) comprising the gas6 DNA to be mutated. The codon(s) in the gas6 DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the gas6 DNA. The plasmid DNA is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures, wherein the two strands of the oligonucleotide are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 5' and 3' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated gas6 DNA sequence.

Covalent modifications of gas6 molecules also are included within the scope of this invention. For example, covalent modifications are introduced into gas6 by reacting targeted amino acid residues of the gas6

with an organic derivatizing agent that is capable of reacting with selected amino acid side chains or the N- or C-terminal residues.

CysteinyI residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. CysteinyI residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

LysinyI and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyI residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4-pentanedione; and transaminase-catalyzed reaction with glyoxylate.

ArginyI residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ^{125}I or ^{131}I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method described above being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides ($R'-N=C=N-R'$), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyI and glutaminyI residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking gas6 to a water-insoluble support matrix or surface for diagnostic and/or therapeutic use. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azido-salicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propionimide yield photoactivatable intermediates that are capable of forming

crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

5 Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains, acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. Creighton, Proteins: Structure and Molecular Properties, pp.79-86 (W.H. Freeman & Co., 1983). Gas6 also is covalently linked to nonproteinaceous polymers, e.g. polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,179,337; 4,301,144; 4,496,689; 4,640,835; 4,670,417; or 4,791,192.

The preferred gas6 is one which is "non-immunogenic in a human" which means that upon contacting the polypeptide in a pharmaceutically acceptable carrier and in a therapeutically effective amount with the appropriate tissue of a human, no state of sensitivity or resistance to the polypeptide is demonstrable upon the second administration of the polypeptide after an appropriate latent period (e.g., 8 to 14 days).

A preferred gas6 variant is one which is essentially not " γ carboxylated" or is less carboxylated than "native" gas6 derived from an endogenous source of the molecule (e.g. serum) or native gas6 made by a recombinant cell wherein the conditions for culturing such a cell facilitate γ carboxylation of the gas6 (e.g. Vitamin K is present in the culture media). Vitamin K is a cofactor for the carboxylase enzyme. The A domain of native gas6 has several glutamic acid residues which are normally γ carboxylated (see Manfioletti *et al.*, *supra*). Accordingly, a convenient way to make a non- γ -carboxylated variant gas6 is to generate gas6 variants which lack one or more of the E residues from the A domain of native gas6 (see Fig. 2) or other gas6 fragments which lack this domain. The extent of γ carboxylation can be measured by amino acid sequence analysis or the barium chloride assay described in Example 11.

"gas6 antagonist" or "antagonist" refers to a substance that opposes or interferes with a functional activity of gas6. Examples of gas6 antagonists include neutralizing antibodies, Rse-IgG, Rse extracellular domain (Rse ECD), Axl-IgG, Axl ECD, Mer-IgG and Mer ECD.

The term "antibody" is used in the broadest sense and specifically covers single anti-gas6 monoclonal antibodies (including agonist and antagonist antibodies) and anti-gas6 antibody compositions with polypeptidic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional

(polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-gas6 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity. (See, e.g. US Pat No 4,816,567 and Mage & Lamoyi, in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc., New York [1987])).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty *et al.*, *Nature* 348:552-554 (1990), for example.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementarity determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin.

The term "neutralizing antibody" as used herein refers to an antibody that is capable of specifically binding to gas6, and which is capable of substantially inhibiting or eliminating the functional activity of gas6 *in vivo* and/or *in vitro*. Typically a neutralizing antibody will inhibit the functional activity of gas6 at least about 50%, and preferably greater than 80%, as determined, for example, by KIRA ELISA (see Example 4 below).

Polyclonal antibodies directed toward gas6 generally are raised in animals by multiple subcutaneous or intraperitoneal injections of gas6 and an adjuvant. It may be useful to conjugate gas6 or a peptide fragment thereof to a carrier protein that is immunogenic in the species to be immunized, such as keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor, using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (conjugation through lysine residues), glutaraldehyde, succinic anhydride, SOCl₂, or R'N = C = NR, where R and R' are different alkyl groups.

Animals are immunized with such gas6-carrier protein conjugates combining 1 mg or 1 µg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5th to 1/10th the original amount of conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is assayed for anti-gas6 antibody titer. Animals are boosted until the antibody titer plateaus. Preferably, the animal is boosted by injection with a conjugate of the same gas6 with a different carrier protein and/or through a different cross-linking agent. Conjugates of gas6 and a suitable carrier protein also can be made in recombinant cell culture as fusion proteins. Also, aggregating agents such as alum are used to enhance the immune response.

Monoclonal antibodies directed toward gas6 are produced using any method which provides for the production of antibody molecules by continuous cell lines in culture. Examples of suitable methods for preparing monoclonal antibodies include the original hybridoma method of Kohler *et al.*, *Nature* 256:495-497 (1975), and the human B-cell hybridoma method, Kozbor, J., *Immunol.* 133:3001 (1984); Brodeur *et al.*, *Monoclonal Antibody Production Techniques and Applications*, pp.51-63 (Marcel Dekker, Inc., New York, 1987).

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be performed following methods known in the art (Jones *et al.*, *Nature* 321:522-525 [1986]; Riechmann *et al.*, *Nature* 332:323-327 [1988]; and Verhoeven *et al.*, *Science* 239:1534-1536 [1988]), by substituting rodent complementarity-determining regions (CDRs) for the corresponding regions of a human antibody.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, for example, Jakobovits *et al.*, *PNAS* 90:2551-2555 (1993); Jakobovits *et al.*, *Nature* 362:255-258 (1993); and Bruggemann *et al.*, *Year in Immunol.*

7:33 (1993). Human antibodies can also be produced in phage-display libraries. Hoogenboom *et al.*, J. Mol. Biol. 227:381 (1991); and Marks *et al.*, J. Mol. Biol. 222:581 (1991).

The term "immunoadhesin" is used interchangeably with the expressions "gas6-immunoglobulin chimera" ("gas6-Ig"), "Rse-immunoglobulin chimera" ("Rse-Ig") and "Mer-immunoglobulin chimera" ("Mer-Ig") and refers to a chimeric molecule that combines a functionally active gas6 (e.g. the D domain thereof), Rse or Mer (e.g. the ECDs thereof) with an immunoglobulin sequence. The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG₁, IgG₂, IgG₃ or IgG₄ subtypes, IgA, IgE, IgD or IgM, but preferably IgG₁ or IgG₃.

Chimeras constructed from a protein sequence (e.g. Rse or Mer receptor ECD) linked to an appropriate immunoglobulin constant domain sequence (immunoadhesins) are known in the art. Immunoadhesins reported in the literature include fusions of the T cell receptor (Gascoigne *et al.*, PNAS (USA) 84:2936-2940 [1987]); CD4 (Capon *et al.*, Nature 337:525-531 [1989]; Traunecker *et al.*, Nature 339:68-70 [1989]; Zettmeissl *et al.*, DNA Cell Biol. USA 9:347-353 [1990]; and Byrn *et al.*, Nature 344:667-670 [1990]); L-selectin (Watson *et al.*, J. Cell. Biol. 110:2221-2229 [1990]; and Watson *et al.*, Nature 349:164-167 [1991]); CD44 (Aruffo *et al.*, Cell 61:1303-1313 [1990]); CD28 and B7 (Linsley *et al.*, J. Exp. Med. 173:721-730 [1991]); CTLA-4 (Lisley *et al.*, J. Exp. Med. 174:561-569 [1991]); CD22 (Stamenkovic *et al.*, Cell 66:1133-1144 [1991]); and TNF receptor (Ashkenazi *et al.*, PNAS (USA) 88:10535-10539 [1991]).

The simplest and most straightforward immunoadhesin design combines the functionally active region(s) of the "adhesin" protein with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the gas6-, Mer-, or Rse-immunoglobulin chimeras of the present invention, nucleic acid encoding the extracellular domain of Rse or Mer receptor or encoding gas6 (or a fragment thereof) will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the Rse-, Mer- or gas6-immunoglobulin chimeras.

In some embodiments, the Rse-, Mer- or gas6-immunoglobulin chimeras are assembled as monomers, or hetero- or homo-multimers, and particularly as dimers or tetramers, essentially as illustrated in WO 91/08298.

In a preferred embodiment, the gas6 sequence, Rse or Mer receptor extracellular domain sequence is fused to the N-terminus of the Fc domain of immunoglobulin G₁ (IgG₁). It is possible to fuse the entire heavy

chain constant region to the gas6, Mer or Rse receptor sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (*i.e.* residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the Rse or Mer receptor or gas6 amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG₁, IgG₂, or IgG₃ heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the Rse-, Mer- or gas6-immunoglobulin chimeras are assembled as multimers, and particularly as homo-dimers or tetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Alternatively, the Rse, Mer or gas6 sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the Rse, Mer or gas6 sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom *et al.*, *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to a Rse, Mer or gas6-immunoglobulin heavy chain fusion polypeptide, or directly fused to the Rse or Mer receptor or gas6. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the Rse-, Mer- or gas6-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567.

In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG₁ and IgG₂ immunoglobulin sequences is preferred. A major advantage of using IgG₁ is that IgG₁ immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG₂ requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG₁ hinge is longer and more flexible, so it can accommodate larger "adhesin" domains that may not fold or function properly when fused to IgG₁. Another consideration may be valency; IgG immunoadhesins are bivalent homodimers, whereas Ig subtypes like IgA and

- IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. For Rse-, Mer- or gas6-immunoadhesins designed for *in vivo* application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG₁, IgG₂ and IgG₄ all have *in vivo* half-lives of 21 days, their relative potencies at activating the complement system are different. IgG₄ does not activate complement, and IgG₂ is significantly weaker at complement activation than IgG₁. Moreover, unlike IgG₁, IgG₂ does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG₃ is optimal for complement activation, its *in vivo* half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG₁ has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fc region; and one of these sites, G1m1, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG₂, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a γ 3 immunoadhesin is greater than that of a γ 1 immunoadhesin.
- Gas6, Mer and Rse immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the gas6, Mer or Rse portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g. Gascoigne *et al.*, *supra*; Aruffo *et al.*, *Cell* 61:1303-1313 [1990]; and Stamenkovic *et al.*, *Cell* 66:1133-1144 [1991]). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For expression in mammalian cells, pRK5-based vectors (Schall *et al.*, *Cell* 61:361-370 [1990]) and CDM8-based vectors (Seed, *Nature* 329:840 [1989]) are useful. The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller and Smith, *Nucleic Acids Res.* 10:6487 [1982]; and Capon *et al.*, *Nature* 337:525-531 [1989]). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36- to 48-mers. Alternatively, PCR techniques can be used to join the two parts of the molecule in-frame with an appropriate vector.
- The choice of host cell line for the expression of the immunoadhesin depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus E1A-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo *et al.*, *Cell* 61:1303-1313 [1990]; and Zettmeissl *et al.*, *DNA Cell Biol.*

(US) 9:347-353 [1990]). If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture. These clones are grown in the presence of increasing levels of DHFR inhibitor methotrexate and clones are selected in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells. For example, components such as light chain or J chain may be provided by certain myeloma or hybridoma host cells (Gascoigne *et al.*, *supra*; and Martin *et al.*, *J. Virol.* 67:3561-3568 [1993]).

Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 [1983]). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss *et al.*, *EMBO J.* 5:15671575 [1986]). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human $\gamma 1$ molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

The expression "Rse extracellular domain" or "Rse ECD" when used herein refers to a polypeptide sequence that shares a ligand-binding function of the extracellular domain of the Rse receptor. "Ligand-binding function" refers to the ability of the polypeptide to bind a Rse ligand, such as gas6. Accordingly, it is often not necessary to include the entire extracellular domain since smaller segments are commonly found to be adequate for ligand binding. The term ECD encompasses polypeptide sequences in which the cytoplasmic domain and hydrophobic transmembrane sequence (and, optionally, 1-20 amino acids amino-terminal to the transmembrane domain) of the Rse receptor have been deleted. Generally the ECD of the Rse receptor comprises amino acid residues from about 1-428 of the mature Rse receptor sequence disclosed in Mark *et al.*, *supra*.

The expression "Mer extracellular domain" or "Mer ECD" when used herein refers to a polypeptide sequence that shares a ligand-binding function of the extracellular domain of the Mer receptor. "Ligand-binding function" refers to the ability of the polypeptide to bind a Mer ligand, such as gas6. Accordingly, it may be

unnecessary to include the entire extracellular domain since smaller segments are commonly found to be adequate for ligand binding. The term ECD encompasses polypeptide sequences in which the cytoplasmic domain and hydrophobic transmembrane sequence (and, optionally, 1-20 amino acids amino-terminal to the transmembrane domain) of the Mer receptor have been deleted. Generally the ECD of the Mer receptor
5 comprises amino acid residues from about 1-499 of the mature human Mer receptor sequence disclosed in the GenBank database (Accession Number U08023).

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising functionally active gas6 fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with functional
10 activity of the gas6. The tag polypeptide preferably also is fairly unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least 6 amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues). The epitope tag is generally proved at the amino- or carboxyl- terminus of the gas6. Such epitope tagged forms of the gas6 are desirable, as the presence thereof can be detected using a labelled antibody against the tag
15 polypeptide. Also, provision of the epitope tag enables the gas6 to be readily purified by affinity purification using the anti-tag antibody.

Tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5, (Field *et al.*, Mol. Cell. Biol. 8:2159-2165 [1988]); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, Molecular and Cellular Biology
20 5(12):3610-3616 [1985]); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky *et al.*, Protein Engineering 3(6):547-553 [1990]). Other tag polypeptides have been disclosed. Examples include the Flag-peptide (Hopp *et al.*, BioTechnology 6:1204-1210 [1988]); the KT3 epitope peptide (Martin *et al.*, Science 255:192-194 [1992]); an α -tubulin epitope peptide (Skinner *et al.*, J. Biol. Chem. 266:15163-15166 [1991]); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al.*, Proc. Natl. Acad. Sci. USA 87:6393-
25 6397 [1990]). Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Gas6-tag polypeptide fusions are most conveniently constructed by fusing the cDNA sequence encoding the gas6 portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the gas6-tag polypeptide chimeras of the present
30 invention, nucleic acid encoding the gas6 (or a fragment thereof) will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible.

Epitope tagged gas6 can be conveniently purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached is most often agarose, but other matrices are available [e.g. controlled pore glass or poly(styrenedivinyl)benzene]. The epitope tagged gas6 can be eluted from
35 the affinity column by varying the buffer pH or ionic strength or adding chaotropic agents, for example.

An "exogenous" compound is defined herein to mean a compound that is foreign to a cell and/or mammal to be treated with the compound, or homologous to a compound found in the cell or mammal but produced outside the cell or mammal.

"Isolated", when used to describe the various proteins disclosed herein, means protein that has been
5 identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-
10 PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain.

"Essentially pure" protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. "Essentially homogeneous" protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

15 Mammalian "Rse receptors" or "Rse receptor protein tyrosine kinases" (*i.e.* "rPTKs") have been described by Mark *et al.* in J. Biol. Chem. 269: 10720 (1994). When used throughout this application, the expression "Rse receptor" refers to endogenous Rse receptor present in a cell of interest as well as Rse receptor which is present in a cell by virtue of the cell having been transformed with nucleic acid encoding the Rse receptor, for example. Accordingly, the Rse receptor may be an amino acid or covalent variant of one of the
20 native Rse receptors described by Mark *et al.*, provided it is still "functionally active" (*i.e.* is able to be activated by a Rse ligand such as gas6). The preferred Rse receptor is endogenous human Rse receptor present in the cell membrane of a human cell.

The phrase "activating Rse receptor" refers to the step of causing the intracellular kinase domain of the Rse receptor to phosphorylate tyrosine residues in a substrate polypeptide. Often, the tyrosine residues are
25 intrinsic to the Rse receptor (*i.e.* the "substrate" comprises the intracellular domain of the Rse receptor). Therefore, the degree of activation correlates with Rse receptor "autophosphorylation". Rse receptor autophosphorylation can be detected by Western blotting using an anti-phosphotyrosine antibody (see Example 3) or by KIRA ELISA (see Example 4). However, activation of the Rse receptor may correlate with phosphorylation of a substrate other than the Rse receptor (*e.g.* a tyrosine kinase existing adjacent the Rse
30 receptor). This can be detected by measuring tyrosine phosphorylation of the substrate (*e.g.* by Western blotting).

Mammalian "Mer receptors" have been described in Graham *et al.*, Cell Growth Differ. 5:647 (1994) (see GenBank database Accession Number U08023 for the correct human Mer sequence) and Graham *et al.*, Oncogene 10(12):2349-2359 (1995). When used throughout this application, the expression "Mer receptor"
35 refers to endogenous Mer receptor present in a cell of interest as well as Mer receptor which is present in a cell

by virtue of the cell having been transformed with nucleic acid encoding the Mer receptor, for example. The preferred Mer receptor is endogenous human Mer receptor present in a human cell.

The phrase "activating Mer receptor" refers to the step of causing the intracellular kinase domain of the Mer receptor to phosphorylate tyrosine residues in a substrate polypeptide. Often, the tyrosine residues are intrinsic to the Mer receptor (*i.e.* the "substrate" comprises the intracellular domain of the Mer receptor). Therefore, the degree of activation correlates with Mer receptor "autophosphorylation". Mer receptor autophosphorylation can be detected by Western blotting using an anti-phosphotyrosine antibody or by KIRA ELISA (see below). However, activation of the Mer receptor may correlate with phosphorylation of a substrate other than the Mer receptor (*e.g.* a tyrosine kinase existing adjacent the Mer receptor). This can be detected by measuring tyrosine phosphorylation of the substrate (*e.g.* by Western blotting).

The expression "enhancing survival of a cell" refers to the act of increasing the period of existence of a cell, relative to an untreated cell which has not been exposed to gas6, either *in vitro* or *in vivo*.

The phrase "enhancing proliferation of a cell" encompasses the step of increasing the extent of growth and/or reproduction of the cell, relative to an untreated cell, either *in vitro* or *in vivo*. An increase in cell proliferation in cell culture can be detected by counting the number of cells before and after exposure to gas6 (see Example 9 herein). The extent of proliferation can be quantified via microscopic examination of the degree of confluency. Cell proliferation can also be quantified by measuring ³H uptake by the cells.

By "enhancing differentiation of a cell" is meant the act of increasing the extent of the acquisition or possession of one or more characteristics or functions which differ from that of the original cell (*i.e.* cell specialization). This can be detected by screening for a change in the phenotype of the cell (*e.g.* identifying morphological changes in the cell, see Example 9 below).

"Physiologically acceptable" carriers, excipients, or stabilizers are ones which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

The terms "treating", "treatment", and "therapy" refer to curative therapy, prophylactic therapy, and preventative therapy.

The term "mammal" refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accord with conventional practice.

2. Gas6 Production

Techniques suitable for the production of native gas6 or gas6 variants are well known in the art and include isolating gas6 from an endogenous source of this polypeptide (e.g. from serum), peptide synthesis (using a peptide synthesizer) and recombinant techniques (or any combination of these techniques). The preferred technique for production of native gas6 or a gas6 variant is a recombinant technique. The preferred gas6 variants are those which are essentially not γ carboxylated. This can be achieved in a number of ways but most conveniently involves creating a molecule which lacks one or more of the glutamic acid residues in the A domain of native gas6 which are normally γ carboxylated. Optionally, the entire A domain may be removed from the native molecule by enzymatic cleavage, but normally a nucleic acid molecule will be isolated which encodes the desired fragment (e.g. the D domain or a G domain therefrom). This nucleic acid molecule can be derived from the native gas6 nucleic acid.

Nucleic acid encoding native gas6 can be isolated from a cDNA library prepared from tissue believed to possess the polypeptide mRNA and to express it at a detectable level (e.g. brain tissue, see Example 6 below). Libraries are screened with probes (such as antibodies or oligonucleotides of about 20-80 bases) designed to identify the gas6 gene or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10-12 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989).

Techniques for generating gas6 mutants via modification of the wildtype nucleic acid have been discussed above. The nucleic acid (e.g., cDNA or genomic DNA) encoding the native gas6 or gas6 variant is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors

are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

The gas6 polypeptide may be produced as a fusion polypeptide with a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells, the signal sequence may be substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders, the latter described in U.S. Pat. No. 5,010,182 issued 23 April 1991), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native gas6 signal sequence is satisfactory, although other mammalian signal sequences may be suitable as well as viral secretory leaders, for example, the herpes simplex gD signal. The DNA for such precursor region is ligated in reading frame to DNA encoding the native gas6/gas6 variant.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*. One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin (Southern *et al.*, *J. Molec. Appl. Genet.* 1:327 [1982]), mycophenolic acid (Mulligan *et al.*, *Science* 209:1422 [1980]) or hygromycin (Sugden *et al.*, *Mol. Cell. Biol.* 5:410-413 [1985]). The three examples given above employ bacterial genes under eukaryotic control to

convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the *gas6* nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes *gas6* variant. Increased quantities of *gas6* are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding the *gas6*. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding *gas6*, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb *et al.*, Nature 282:39 [1979]; Kingsman *et al.*, Gene 7:141 [1979]; or Tschemper *et al.*, Gene 10:157 [1980]). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics 85:12 [1977]). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi *et al.*, Curr. Genet. 12:185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, Bio/Technology

8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*, *Bio/Technology* 9:968-975 (1991).

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the gas6 nucleic acid. A large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to gas6-encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems (Chang *et al.*, *Nature* 275:615 [1978]; and Goeddel *et al.*, *Nature* 281:544 [1979]), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057 [1980] and EP 36,776) and hybrid promoters such as the tac promoter (deBoer *et al.*, *Proc. Natl. Acad. Sci. USA* 80:21-25 [1983]). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding the gas6 (Siebenlist *et al.*, *Cell* 20:269 [1980]) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the gas6.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, *J. Biol. Chem.* 255:2073 [1980]) or other glycolytic enzymes (Hess *et al.*, *J. Adv. Enzyme Reg.* 7:149 [1968]; and Holland, *Biochemistry* 17:4900 [1978]), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytichrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman *et al.*, EP 73,657A. Yeast enhancers also are advantageously used with yeast promoters.

Gas6 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July

1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter or from heat-shock promoters.

- The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers *et al.*, *Nature* 273:113 (1978); Mulligan and Berg, *Science* 209:1422-1427 (1980); Pavlakis *et al.*, *Proc. Natl. Acad. Sci. USA* 78:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a *HindIII* E restriction fragment. Greenaway *et al.*, *Gene* 18:355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Gray *et al.*, *Nature* 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Cansani and Berg, *Proc. Natl. Acad. Sci. USA* 79:5166-5170 (1982) on expression of the human interferon β 1 gene in cultured mouse and rabbit cells; and Gorman *et al.*, *Proc. Natl. Acad. Sci. USA* 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

- Transcription of DNA encoding the *gas6* by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are relatively orientation and position independent, having been found 5' (Laimins *et al.*, *Proc. Natl. Acad. Sci. USA* 78:993 [1981]) and 3' (Lusky *et al.*, *Mol. Cell Bio.* 3:1108 [1983]) to the transcription unit, within an intron (Banerji *et al.*, *Cell* 33:729 [1983]), as well as within the coding sequence itself (Osborne *et al.*, *Mol. Cell Bio.* 4:1293 [1984]). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the *gas6*-encoding sequence, but is preferably located at a site 5' from the promoter.

- Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the *gas6*.

Construction of suitable vectors containing one or more of the above listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing *et al.*, Nucleic Acids Res. 9:309 (1981) or by the method of Maxam *et al.*, Methods in Enzymology 65:499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding gas6. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sambrook *et al.*, *supra*, pp. 16.17 - 16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of gas6 variants having desired binding specificities/affinities.

Other methods, vectors, and host cells suitable for adaptation to the synthesis of the gas6 in recombinant vertebrate cell culture are described in Gething *et al.*, Nature 293:620-625 (1981); Mantei *et al.*, Nature 281:40-46 (1979); Levinson *et al.*; EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of gas6 is pRK5 (EP 307,247) or pSV16B (PCT pub. no. WO 91/08291 published 13 June 1991).

The choice of host cell line for the expression of gas6 depends mainly on the expression vector. Where it is desired to make a gas6 variant which is essentially not γ carboxylated, it may be desirable to select a host cell which does not have γ carboxylase enzyme, especially where the nucleic acid encoding gas6 also encodes the A domain thereof. Often, a useful host for this purpose is a non-mammalian cell (e.g. a prokaryotic cell known to be deficient in this enzyme). Alternatively a mammalian cell line can be utilized which has been made deficient in this enzyme.

Suitable host cells for cloning or expressing the vectors herein are prokaryote, yeast, or other higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

Strain W3110 is a particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 27C7. The complete genotype of 27C7 is *tonAΔ ptr3*
 5 *phoAΔE15 Δ(argF-lac)169 ompTΔ degP41karf*. Strain 27C7 was deposited on 30 October 1991 in the American Type Culture Collection as ATCC No. 55,244. Alternatively, the strain of *E. coli* having mutant periplasmic protease disclosed in U.S. Patent No. 4,946,783 issued 7 August 1990 may be employed. Alternatively, methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning
 10 or expression hosts for gas6-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach and Nurse, Nature 290:140 [1981]; EP 139,383 published May 2, 1985); *Kluyveromyces* hosts (U.S. Patent No. 4,943,529; Fleer *et al.*, *supra*) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt *et al.*, J. Bacteriol. 737
 15 [1983]), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilum* (ATCC 36,906; Van den Berg *et al.*, *supra*), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna *et al.*, J. Basic Microbiol. 28:265-278 [1988]); *Candida*; *Trichoderma reesei* (EP 244,234); *Neurospora crassa* (Case *et al.*, Proc. Natl. Acad. Sci. USA 76:5259-5263 [1979]); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31
 20 October 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published 10 January 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance *et al.*, Biochem. Biophys. Res. Commun. 112:284-289 [1983]; Tilburn *et al.*, Gene 26:205-221 [1983]; Yelton *et al.*, Proc. Natl. Acad. Sci. USA 81:1470-1474 [1984]) and *A. niger* (Kelly and Hynes, EMBO J. 4:475-479 [1985]).

Suitable host cells for the expression of glycosylated gas6 are derived from multicellular organisms.
 25 Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, e.g.,
 30 Luckow *et al.*, Bio/Technology 6:47-55 (1988); Miller *et al.*, in Genetic Engineering, Setlow *et al.*, eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda *et al.*, Nature 315:592-594 (1985). A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the gas6 DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the gas6 is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the gas6 DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker *et al.*, J. Mol. Appl. Gen. 1:561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published 21 June 1989.

- 10 Propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, Academic Press, Kruse and Patterson, editors [1973]). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, J. Gen. Virol. 36:59 [1977]); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 [1980]); mouse sertoli cells (TM4, Mather, Biol. Reprod. 23:243-251 [1980]); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRJ cells (Mather *et al.*, 20 Annals N.Y. Acad. Sci. 383:44-68 [1982]); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected with the above-described expression or cloning vectors of this invention and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Depending on the host cell used, transfection is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sambrook *et al.*, *supra*, or electroporation is generally used for prokaryotes or other 25 cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw *et al.*, Gene 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

- 30 For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Patent No. 4,399,216 issued 16 August 1983. Transformations into yeast are typically carried out according to the method of Van Solingen *et al.*, J. Bact. 130:946 (1977) and Hsiao *et al.*, Proc. Natl. Acad. Sci. (USA) 76:3829 (1979). However, other methods for 35 introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with

intact cells, or polycations, e.g., polybrene, polyornithine, etc., may also be used. For various techniques for transforming mammalian cells, see Keown *et al.*, Methods in Enzymology (1989), Keown *et al.*, Methods in Enzymology 185:527-537 (1990), and Mansour *et al.*, Nature 336:348-352 (1988).

5 Prokaryotic cells used to produce the gas6 polypeptide of this invention are cultured in suitable media as described generally in Sambrook *et al.*, *supra*.

The mammalian host cells used to produce the gas6 of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium (MEM), Sigma, RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium (DMEM), Sigma are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace, Meth. Enz. 58:44 (1979), Barnes and Sato, Anal. Biochem. 102:255 (1980), U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Patent Re. 30,985; or U.S. Patent No. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art.

In certain embodiments, it is desirable to culture the transformed host cells in the absence of Vitamin K as this can reduce γ carboxylation of the A domain of the gas6 polypeptide. Alternatively, the transformed host cells can be cultured in the presence of a carboxylase inhibitor, such as warfarin

The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed., IL Press, 1991. The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

Gas6 preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates.

When gas6 is produced in a recombinant cell other than one of human origin, it is completely free of proteins or polypeptides of human origin. However, it is necessary to purify gas6 from cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to gas6. As a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration; optionally, the protein may be concentrated with a commercially available protein concentration filter, followed by separating the gas6 from other impurities by one or more steps selected from heparin Sepharose chromatography, immunoaffinity chromatography, ion-exchange column fractionation (e.g., on DEAE or

matrices containing carboxymethyl or sulfopropyl groups), chromatography on Blue-Sepharose, CM Blue-Sepharose, MONO-Q, MONO-S, lentil lectin-Sepharose, WGA-Sepharose, Con A-Sepharose, Ether Toyopearl, Butyl Toyopearl, Phenyl Toyopearl, or protein A Sepharose, SDS-PAGE chromatography, silica chromatography, chromatofocusing, reverse phase HPLC (e.g., silica gel with appended aliphatic groups), gel
5 filtration using, e.g., Sephadex molecular sieve or size-exclusion chromatography, chromatography on columns that selectively bind the gas6, and ethanol or ammonium sulfate precipitation. A protease inhibitor may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants. Examples of suitable protease inhibitors include phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride-bestatin,
10 chymostatin, and benzamidin.

Gas6 variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native gas6, taking account of any substantial changes in properties occasioned by the variation. For example, preparation of "epitope tagged" gas6 facilitates purification using an immunoaffinity column containing antibody to the antigen to adsorb the fusion polypeptide. Immunoaffinity columns such as a rabbit polyclonal
15 anti-gas6 column can be employed to absorb the gas6 variant by binding it to at least one remaining immune epitope. One skilled in the art will appreciate that purification methods suitable for native gas6 may require modification to account for changes in the character of gas6 or its variants upon production in recombinant cell culture.

3. In Vitro and In Vivo Uses for Gas6

20 The present invention provides methods for activating Rse receptor or Mer receptor and/or enhancing survival, proliferation or differentiation of cells comprising the Rse or Mer receptor using gas6. The gas6 useful in the practice of the present invention can be prepared in a number of ways which have been described in the previous section (see also Example 6 below).

The gas6 may be from a human or any non-human species. For instance, a mammal may be treated with
25 gas6 from a different mammalian species (e.g., mice can be treated with human gas6). There is substantial homology (about 81% amino acid identity) between murine gas6 and human gas6, and thus, it is expected that gas6 from different mammalian species can be employed. Preferably, however, the mammal is treated with homologous gas6 (e.g., humans are treated with human gas6) to avoid potential immunogenicity of the gas6 in the mammal.

30 The present invention includes methods of activating Rse or Mer receptor and/or enhancing survival, proliferation or differentiation of cells comprising the Rse or Mer receptor *in vivo* and *in vitro*. Normally, the cells will be treated with the gas6 polypeptide. However, gene therapy approaches have been described in the art and are encompassed by the present invention. These techniques include gene delivery to a cell using adenovirus, herpes simplex 1 virus or adeno-associated virus as well as lipid-based delivery systems (e.g.

liposomes). Retroviruses are useful for *ex vivo* gene therapy approaches. Accordingly, it is possible to administer the nucleic acid encoding gas6, resulting in expression of the gas6 polypeptide in the patient or in tissue culture. For exemplary gene therapy techniques see WO 93/25673 and the references cited therein.

- In accordance with the *in vitro* methods of the invention, cells comprising the Rse or Mer receptor are provided and placed in a cell culture medium. Examples of Rse-receptor-containing cells include neural cells, e.g., brain cells (such as neurons of the neocortex, cerebellum and hippocampus); glial cells (e.g. Schwann cells or astrocytes); kidney or breast-derived cells; cells derived from the ovary or testes; fibroblast cells such as mouse 3T3 cells; cells from the hematopoietic system such as CMK11-5. Examples of Mer-receptor-containing cells include peripheral blood mononuclear cells, bone marrow mononuclear cells, monocytes, primary hematopoietic cells and cells derived from testis, ovary, prostate, lung, kidney, spleen, peripheral blood leukocyte, placenta, thymus, small intestine, colon or liver. Exemplary cell lines to be cultured using gas6 include T lymphocyte leukemia cell lines (e.g. CCRF-HSB-2, JURKAT, HPB-ALL and Peer); K-562 cell line; monocytic leukemia/lymphoma cell lines (such as U-937); megakaryoblastic leukemia cell lines (e.g. UT-7) and other cell lines which express Mer receptor as described in Graham *et al.*, *Cell Growth Differ.* 5:647 (1994).
- Suitable tissue culture media are well known to persons skilled in the art and include, but are not limited to, Minimal Essential Medium (MEM), RPMI-1640, and Dulbecco's Modified Eagle's Medium (DMEM). These tissue culture medias are commercially available from Sigma Chemical Company (St. Louis, MO) and GIBCO (Grand Island, NY). The cells are then cultured in the cell culture medium under conditions sufficient for the cells to remain viable and grow in the presence of an effective amount of gas6. The cells can be cultured in a variety of ways, including culturing in a clot, agar, or liquid culture.

- The cells are cultured at a physiologically acceptable temperature such as 37°C, for example, in the presence of an effective amount of gas6. The amount of gas6 may vary, but preferably is in the range of about 10 ng/ml to about 1 mg/ml. The gas6 can of course be added to the culture at a dose determined empirically by those in the art without undue experimentation. The concentration of gas6 in the culture will depend on various factors, such as the conditions under which the cells and gas6 are cultured. The specific temperature and duration of incubation, as well as other culture conditions, can be varied depending on such factors as, e.g., the concentration of the gas6, and the type of cells and medium. Those skilled in the art will be able to determine operative and optimal culture conditions without undue experimentation. Proliferation, differentiation and/or survival of the cells (e.g. neurons or mononuclear cells) in the cultures can be determined by various assays known in the art such as those described above.

- It is contemplated that using gas6 to enhance cell survival, growth and/or differentiation *in vitro* will be useful in a variety of ways. For instance, neural cells cultured *in vitro* in the presence of gas6 can be infused into a mammal suffering from reduced levels of the cells. In other embodiments, gas6 may be used to culture hematopoietic cells (such as monocytes/macrophages) *ex vivo* which can be administered to a patient having decreased levels of these blood cells (for example where the patient has undergone chemo- or radiation therapy).

Stable *in vitro* cultures can also be used for isolating cell-specific factors and for expression of endogenous or recombinantly introduced proteins in the cell. Gas6 may also be used to enhance cell survival, proliferation and/or differentiation of cells which support the growth and/or differentiation of other cells in cell culture (e.g. stromal cells supporting bone marrow non-adherent cells). In this manner, Schwann cells may promote neuronal survival in cell culture.

Gas6 is considered to be particularly useful for growing Schwann cells *ex vivo*. It is desirable to have such populations of cells in cell culture for isolation of cell-specific factors e.g. P75^{NQF} which is a Schwann cell specific marker. Such factors are useful as diagnostic tools or, in the case of P75^{NQF}, can be used as antigens to generate antibodies for diagnostic use. It is also desirable to have stable populations of Schwann cells in cell culture to facilitate characterization of other mitogens and growth inhibitory agents for these cells.

The invention also provides *in vivo* uses for gas6. Based on the ability of gas6 to promote proliferation of glial cells (see Example 9), it is believed that this molecule will be particularly useful for treating diseases which involve demyelination, damage or loss of glial cells (e.g. multiple sclerosis).

Gas6 is also believed to be useful in promoting the development, maintenance, and/or regeneration of neurons *in vivo*, including central (brain and spinal chord), peripheral (sympathetic, parasympathetic, sensory, and enteric neurons), and motoneurons. Accordingly, gas6 may be utilized in methods for the diagnosis and/or treatment of a variety of "neurologic diseases or disorders" which effect the nervous system of a mammal, such as a human.

Such diseases or disorders may arise in a patient in whom the nervous system has been damaged by, e.g., trauma, surgery, stroke, ischemia, infection, metabolic disease, nutritional deficiency, malignancy, or toxic agents. The agent is designed to promote the survival or growth of neurons. For example, gas6 can be used to promote the survival or growth of motoneurons that are damaged by trauma or surgery. Also, gas6 can be used to treat motoneuron disorders, such as amyotrophic lateral sclerosis (Lou Gehrig's disease), Bell's palsy, and various conditions involving spinal muscular atrophy, or paralysis. Gas6 can be used to treat human "neurodegenerative disorders", such as Alzheimer's disease, Parkinson's disease, epilepsy, multiple sclerosis, Huntington's chorea, Down's Syndrome, nerve deafness, and Meniere's disease.

Further, gas6 can be used to treat neuropathy, and especially peripheral neuropathy. "Peripheral neuropathy" refers to a disorder affecting the peripheral nervous system, most often manifested as one or a combination of motor, sensory, sensorimotor, or autonomic neural dysfunction. The wide variety of morphologies exhibited by peripheral neuropathies can each be attributed uniquely to an equally wide number of causes. For example, peripheral neuropathies can be genetically acquired, can result from a systemic disease, or can be induced by a toxic agent. Examples include but are not limited to distal sensorimotor neuropathy, or autonomic neuropathies such as reduced motility of the gastrointestinal tract or atony of the urinary bladder. Examples of neuropathies associated with systemic disease include post-polio syndrome; examples of hereditary neuropathies include Charcot-Marie-Tooth disease, Refsum's disease, Abetalipoproteinemia, Tangier disease,

Krabbe's disease, Metachromatic leukodystrophy, Fabry's disease, and Dejerine-Sottas syndrome; and examples of neuropathies caused by a toxic agent include those caused by treatment with a chemotherapeutic agent such as vincristine, cisplatin, methotrexate, or 3'-azido-3'-deoxythymidine.

Given expression of Rse receptor and Mer receptor on hematopoietic cells, gas6 may be used to enhance
5 repopulation of mature blood cell lineages in patients having undergone chemo- or radiation therapy or bone marrow transplantation therapy. It is contemplated that gas6 will act via an enhancement of the proliferation and/or differentiation of hematopoietic cells (e.g. monocytes and megakaryocytes). Gas6 may similarly be useful for treating diseases characterized by a decrease in blood cells. Examples of these diseases include anemia (including macrocytic and aplastic anemia); thrombocytopenia; monocytopenia; hypoplasia; immune
10 (autoimmune) thrombocytopenic purpura (ITP); and HIV induced ITP. Gas6 may also be used to promote growth and/or repair of tissues (e.g. testis, ovary, prostate, lung or kidney) which express either, or both, of these receptors. Also, gas6 may be used to improve reproductive function, given high level expression of Mer receptor in the testes and ovaries.

In that Mer is expressed on mononuclear cells, it is contemplated that gas6 may be used to treat
15 conditions in which proliferation and/or differentiation of these cells is desired. For example, gas6 may be used to increase monocyte (e.g. macrophage) levels in a patient where this is required or desired.

In other embodiments, gas6 may be used to modulate function of cells possessing the Mer or Rse receptor. For example, gas6 may be used to activate monocytes/macrophages in situations where such activation is desired (e.g. to treat infections).

In still further embodiments of the invention, gas6 antagonists, and especially anti-gas6 antibodies, can be administered to patients suffering from diseases or disorders (e.g., neurologic diseases or disorders) characterized by excessive production of gas6 and/or excessive Rse or Mer receptor activation by gas6. Gas6 antagonist may be used in the prevention of aberrant regeneration of sensory neurons such as may occur post-operatively, or in the selective ablation of sensory neurons, for example, in the treatment of chronic pain
25 syndromes. Gas6 antagonists may also be used to treat monocytosis or malignancies characterized by excessive Rse or Mer receptor activation (such as lymphoid malignancies) or to treat monocyte/macrophage-mediated inflammation.

Therapeutic formulations of gas6 and gas6 antagonist are prepared by mixing gas6 or gas6 antagonist, having the desired degree of purity, with optional pharmaceutically acceptable carriers, excipients, or stabilizers
30 which are well known. Acceptable carriers, excipients or stabilizers are nontoxic to the patient at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other
35 carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as

mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

It may be desirable to adsorb gas6 onto a membrane, such as a silastic membrane, which can be implanted in proximity to damaged neural tissue, or to incorporate gas6 into liposomes. PCT Pub. No. WO 91/04014 (published April 4, 1991). In another embodiment, the gas6 used for therapeutic effect is gas6

covalently joined to another protein, such as an immunoglobulin domain (for example, to produce gas6-IgG).

Gas6 optionally is combined with or administered in concert with other neurotrophic factors to achieve a desired therapeutic effect. For example, gas6 may be used together with nerve growth factor (NGF), neurotrophins (NT-3), bone derived nerve factor (BDNF), neurotrophins-4 and -5 (NT-4/5), an insulin-like growth factor (e.g., IGF-1 or IGF-2) or another neurotrophic factor to achieve a synergistic stimulatory effect on the growth of sensory neurons, wherein the term "synergistic" means that the effect of the combination of gas6 with a second substance is greater than that achieved with either substance used individually.

For use in hematopoiesis, gas6 may be administered in concert with one or more cytokines. Included among the cytokines are growth hormone, insulin-like growth factors, human growth hormone, N-methionyl human growth hormone, bovine growth hormone, parathyroid hormone, thyroxine, insulin, proinsulin, relaxin, prolrelaxin, glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH), hematopoietic growth factor, hepatic growth factor, fibroblast growth factor, prolactin, placental lactogen, tumor necrosis factor- α and - β , mullerian-inhibiting substance, mouse gonadotropin-associated peptide, inhibin, activin, vascular endothelial growth factor, integrin, thrombopoietin, nerve growth factors such as NGF- β , platelet-growth factor, transforming growth factors (TGFs) such as TGF- α and TGF- β , insulin-like growth factor-I and -II, erythropoietin (EPO), osteoinductive factors, interferons such as interferon- α , - β , and - γ , colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF), granulocyte-macrophage-CSF (GM-CSF), and granulocyte-CSF (G-CSF), interleukins (ILs) such as IL-1, IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12 and other polypeptide factors including LIF, SCF, and kit-ligand.

As used herein the foregoing terms are meant to include proteins from natural sources or from recombinant cell culture. Similarly, the terms are intended to include biologically active equivalents; e.g., differing in amino acid sequence by one or more amino acids or in type or extent of glycosylation.

Gas6 and gas6 antagonist to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration of a solution of gas6 or gas6 antagonist through sterile filtration membranes. Thereafter, the filtered solution may be placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The filtered solution also may be lyophilized to produce sterile gas6 or gas6 antagonist in a powder form.

Methods for administering gas6 and gas6 antagonists *in vivo* include injection or infusion by intravenous, intraperitoneal, intracerebral, intrathecal, intramuscular, intraocular, intraarterial, or intralesional routes, and by means of sustained-release formulations.

Sustained-release formulations generally consist of gas6 or gas6 antagonist and a matrix from which the gas6 or gas6 antagonist are released over some period of time. Suitable matrices include semipermeable polymer matrices in the form of shaped articles, for example, membranes, fibers, or microcapsules. Sustained release matrices may comprise polyesters, hydrogels, polylactides, U.S. Pat. No. 3,773,919, copolymers of L-glutamic acid and γ -ethyl-L-glutamate, Sidman *et al.*, Biomaterials 22:547-556 (1983), poly (2-hydroxyethyl-methacrylate), or ethylene vinyl acetate, Langer *et al.*, J. Biomed. Mater. Res. 15:167-277 (1981); and Langer, Chem. Tech. 12:98-105 (1982).

In one embodiment of the invention, the therapeutic formulation comprises gas6 or gas6 antagonist entrapped within or complexed with liposomes. For example, gas6 covalently joined to a glycerophosphatidyl-inositol moiety may be used to form a liposome comprising gas6. In a further embodiment, the therapeutic formulation comprises cells actively producing gas6 or gas6 antagonist. Such cells may be directly introduced into the tissue of a patient, or may be encapsulated within porous membranes which are then implanted in a patient, in either case providing for the delivery of gas6 or gas6 antagonist into areas within the body of the patient in need of increased or decreased concentrations of gas6. Alternatively, an expression vector comprising gas6 DNA may be used for *in vivo* transformation of a patient's cells to accomplish the same result.

An effective amount of gas6 or gas6 antagonist to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. A typical daily dosage might range from about 1 μ g/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Where possible, it is desirable to determine appropriate dosage ranges first *in vitro*, for example, using assays for cell survival or growth which are known in the art, and then in suitable animal models, from which dosage ranges for human patients may be extrapolated. In a specific embodiment of the invention, a pharmaceutical composition effective in promoting the survival or growth of neurons will provide a local gas6 concentration *in vivo* of between about 0.1 and 10 ng/ml.

The invention further provides an article of manufacture and kit containing materials useful for activating the Rse or Mer receptor or enhancing survival, proliferation or differentiation of cells comprising the Rse or Mer receptor. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for activating the Rse or Mer receptor and/or enhancing survival, proliferation and/or differentiation of cells having the receptor of interest. The active agent in the composition is gas6. The label on the container indicates that the composition is used for activating the Rse or Mer receptor and/or enhancing survival, proliferation and/or differentiation of cells having this receptor, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention comprises the container described above and a second container comprising a buffer. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

- The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature citations herein are incorporated by reference.

Example 1

Production of Rse-IgG Fusion Protein

- To identify a source of a Rse ligand (Rse-L), a fusion protein containing the extracellular domain of human Rse followed by the Fc portion of human IgG (Rse-IgG) was used as a probe to screen cells for surface bound Rse-L using flow cytometry (see Example 2 below). Rse-IgG was constructed by fusing the sequence encoding the extracellular domain (amino acids 1-428) of human Rse (Mark *et al.*, Journal of Biological Chemistry 269(14):10720-10728 [1994]) to amino acids 216-443 of human IgG₁ through a BstEII linker (adding amino acids Val and Thr). The linker was added to Rse sequences by PCR using the primers (5'-TCAAGACAAATGGAAACCCAGG [SEQ ID NO: 4] and 5'-CATGGAATTCGGTGACCGATGTGCGGCTGTGAGGAG [SEQ ID NO: 5]). The cDNA encoding Rse-IgG was transferred into an SV40 based expression vector and introduced into DHFR⁺ CHO cells by electroporation (250 volts, 960 μ F). DHFR⁺ cells were selected and Rse-IgG expression in individual clones was determined using a human Fc-specific ELISA. Rse-IgG was purified on a protein A-Sepharose column (Pharmacia).

Example 2

Binding Analysis

- Fluorescence activated cell sorting (FACS) analysis using Rse-IgG was performed as described in Goodwin *et al.*, Cell 73:447 (1993). The megakaryocytic leukemia line CMK11-5 cells (Adachi *et al.*, Exp Hematol, 19:923 [1991]) specifically bound Rse-IgG but not control fusion proteins containing the identical Fc domain such as HGFr-IgG (Mark *et al.*, J. Biol. Chem., 267:26166 [1992]) or CD4-IgG (Capon *et al.*, Nature 337:525 [1989]). Binding of Rse-IgG was increased by the addition of Ca²⁺ and abolished by treatment with 2 mM EDTA.

- Subsequently, an *in vitro* binding assay was established to characterize the interaction of ¹²⁵I-Rse-IgG with the putative cell surface bound Rse-L. CMK11-5 cells were suspended in 10 mM TrisCl, pH 7.5 for 10 min on ice, lysed by a combination of sonication and shearing, and whole membranes collected by centrifugation and stored in 50 mM TrisCl, pH 7.5, 20% glycerol at -80°C. Membranes equivalent to 200,000 cells were combined with fetal bovine serum (FBS) or column fractions, competitors, and ¹²⁵I-Rse-IgG in a total volume

of 0.1-0.12 ml. After a 30 min incubation at room temperature, 1 ml of ice cold assay buffer was added to each tube. Then, the membrane associated radioactivity was collected by centrifugation for 4 min at 15000g, separated from unbound radioactivity by aspiration of the supernatant fluid and counted in a γ counter. The assay buffer was 50 mM Tris-HCl, 0.05% Tween-20, 0.1% BSA, 5 mM CaCl_2 .

- 5 Because flow cytometric analyses were performed in the presence of serum, the effect of FBS in the membrane binding assay was determined. Binding was found to be absolutely dependent on FBS concentration; no displaceable binding was seen in the absence of FBS and half maximum binding was observed with 0.58% FBS (Fig. 3A).

- Binding was also Ca^{2+} -dependent; half maximum binding was obtained with 0.18 mM Ca^{2+} (Fig. 3B).
 10 Although the apparent number of binding sites for Rse-IgG was dependent on the concentration of FBS, the affinity was not greatly changed [K_d of 0.82 nM in 1% FBS vs. 2.2 nM in 10% FBS] (Fig. 3C). Binding was specific; other recombinant IgG fusion proteins, such as CD4-IgG, did not compete for binding with ^{125}I -Rse-IgG.

Example 3

- 15 Epitope-Tagged Rse Receptor and Activation Thereof

Chinese Hamster Ovary (CHO) cells expressing a version of Rse receptor having a Herpes simplex virus type I (HSV-1) C-terminal glycoprotein D (gD) flag (Paborsky *et al.*, Protein Engineering 3(6):547-553 [1990]) were generated in order to further characterize a Rse-L.

- Synthetic double stranded oligonucleotides were used to reconstitute the coding sequence for the C-terminal 10 amino acids (880 - 890) of human Rse and add an additional 21 amino acids containing the gD epitope for the antibody 5B6 (Paborsky *et al.*, *supra*) and a stop codon. The final sequence of the synthetic portion of the fusion gene was:

coding strand:

- 5'-GCAAGGGCTACTGCCACACTCGAGCTGCGCAGATGCTAGCCTCAAGATGGCT G
 25 ATCCAAATCGATTCCGCGCAAGATCTTCCGGTCTGTAGA-3' [SEQ ID NO: 6]

noncoding strand:

5'-AGCTTCTACAGGACCGGAAGATCTTGGCCGGAATCGATTGGATCAGCCATCTT G
 AGGCTAGCATCTGCGCAGCTCGAGTGTGGCAGTAGCCCTTGCTGCA-3' [SEQ ID NO: 7].

- The synthetic DNA was ligated with the cDNA encoding amino acids 1-880 of human Rse at the PstI site beginning at nucleotide 2644 of the published human Rse cDNA sequence (Mark *et al.*, Journal of Biological Chemistry 269(14):10720-10728 [1994]) and HindIII sites in the polylinker of the expression vector pSV7.ID.LL (see PCT/US94/13329) derived from the vector pRK (Suva *et al.*, Science, 237:893-896 [1987]) to create the expression plasmid pSV.ID.Rse.gD. Briefly, the expression plasmid comprises a dicistronic primary transcript which contains sequence encoding DHFR bounded by 5' splice donor and 3' splice acceptor intron

splice sites, followed by sequence that encodes the Rse.gD. The full length (non-spliced) message contains DHFR as the first open reading frame and therefore generates DHFR protein to allow selection of stable transformants.

dp12.CHO cells (EP 307,247 published 15 March 1989) were electroporated with pSV.ID.Rse.gD which had been linearized at a unique NotI site in the plasmid backbone. The DNA was ethanol precipitated after phenol/chloroform extraction and was resuspended in 10 μ l 10/1 Tris/EDTA. Then, 20 μ g of DNA was incubated with 10⁵ CHO.dp12 cells in 1 ml of PBS on ice for 10 min. before electroporation at 350 volts and 330 μ s. Cells were returned to ice for 10 min. before being plated into non-selective medium. After 24 hours cells were fed nucleoside-free medium to select for stable DHFR+ clones.

To identify a cell line that expresses Rse.gD nucleic acid, candidate clones were screened by FACS analysis using the polyclonal antiserum 19B which recognizes epitopes in the extracellular domain of Rse. To confirm that clones that scored positive in the FACS assay express full-length Rse.gD nucleic acid, cell lysates were prepared (Lokker *et al.*, EMBO J, 11:2503-2510 [1992]) and solubilized Rse.gD was immunoprecipitated with the 19B antisera. The immunoprecipitated proteins were fractionated under reducing conditions using 7% PAGE, blotted onto nitrocellulose and then probed with the anti-gD 5B6 antibody (Paborsky *et al.*, *supra*) which was detected with a horseradish peroxidase conjugated anti-mouse IgG antibody.

The ability of Rse.gD in cell clones to undergo autophosphorylation in response to 20% FBS, partially purified fractions of FBS containing the Rse receptor binding activity (*i.e.* 1:10 dilution of the QSE fraction obtained in Example 5 below) or control (*i.e.* no additions) was determined by Western blotting. Briefly, 5 x 10⁵ dp12.CHO cells transformed with Rse.gD nucleic acid as described above were seeded on a 60mm dish in the presence of serum for 6h. The cells were then washed in phosphate buffered saline (PBS) and serum-starved for 16h. The serum starved cells were then exposed to the sample for 10 min. The Rse.gD protein was immunoprecipitated from CHO cell lysates using the anti-gD 5B6 monoclonal antibody. Proteins were fractionated on 7% SDS-PAGE under reducing conditions and transferred to nitrocellulose. Phosphorylation of Rse was detected with labelled anti-phosphotyrosine antibody 4G10 (obtained commercially from UBI, New York).

Addition of either 20% FBS or partially purified fractions of FBS containing the Rse-IgG binding activity to serum-starved cells expressing Rse-gD resulted in phosphorylation of the 140 kDa Rse receptor on tyrosine residues. The Rse receptor was not activated by the control.

Example 4

KIRA ELISA

The activity in FBS that activated Rse.gD was further characterized using an ELISA-based "KIRA" (for Kinase Receptor Activation) assay that allows high-throughput analysis of potential Rse-L sources. See Figure 4 for a schematic representation of this assay.

Rse.gD transformed dp12.CHO cells produced as described in Example 3 were seeded (5×10^4 per well) in the wells of a flat-bottom-96 well culture plate in 100 μ l media and cultured overnight at 37°C in 5% CO₂. The following morning the well supernatants were decanted, and the plates were lightly tamped on a paper towel. 50 μ l of media containing QSE fraction obtained as described in Example 5 below or control (*i.e.* media alone) was then added to each well. For neutralization experiments, potential ligand sources were treated at room temperature for 30 min with Rse-IgG or CD4-IgG (100 μ g/ml) prior to addition to the cells. The cells were stimulated at 37°C for 30 min., the well supernatants were decanted, and the plates were once again lightly tamped on a paper towel. To lyse the cells and solubilize the receptors, 100 μ l of lysis buffer was added to each well. Lysis buffer consisted of 150 mM NaCl containing 50 mM HEPES (Gibco), 0.5 % Triton-X 100 (Gibco), 0.01 % thimerosal, 30 KIU/ml aprotinin (ICN Biochemicals, Aurora, OH), 1mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF; ICN Biochemicals), 50 μ M leupeptin (ICN Biochemicals), and 2 mM sodium orthovanadate (Na₂VO₄; Sigma Chemical Co, St. Louis, MO), pH 7.5. The plate was then agitated gently on a plate shaker (Bellco Instruments, Vineland, NJ) for 60 min. at room temperature.

While the cells were being solubilized, an ELISA microtiter plate (Nunc Maxisorp, Inter Med, Denmark) coated overnight at 4°C with the 5B6 monoclonal anti-gD antibody (0.5 μ g/ml in 50 mM carbonate buffer, pH 9.6, 100 μ l/well) was decanted, tamped on a paper towel and blocked with 150 μ l/well of Block Buffer (PBS containing 0.5 % BSA [Intergen Company, Purchase, NY] and 0.01 % thimerosal) for 60 min. at room temperature with gentle agitation. After 60 minutes, the anti-gD 5B6 coated plate was washed 6 times with wash buffer (PBS containing 0.05 % Tween-20 and 0.01 % thimerosal) using an automated plate washer (ScanWasher 300, Skatron Instruments, Inc, Sterling, VA).

The lysate containing solubilized Rse.gD from the cell-culture microtiter well was transferred (85 μ l/well) to anti-gD 5B6 coated and blocked ELISA well and was incubated for 2 h at room temperature with gentle agitation. The unbound Rse.gD was removed by washing with wash buffer and 100 μ l of biotinylated 4G10 (anti-phosphotyrosine) at 0.15 μ g/ml in buffer (PBS containing 0.5 % BSA, 0.05 % Tween-20, 5 mM EDTA, and 0.01 % thimerosal), was added to each well. After incubation for 2 h at room temperature the plate was washed and 100 μ l of HRPO-conjugated streptavidin (Zymed Laboratories, S. San Francisco, CA) diluted 1:6x10⁴ in dilution buffer was added to each well. The plate was incubated for 30 minutes at room temperature with gentle agitation. The free avidin-conjugate was washed away and 100 μ l freshly prepared substrate solution (tetramethyl benzidine [TMB]; 2-component substrate kit; Kirkegard and Perry, Gaithersburg, MD) was added to each well. The reaction was allowed to proceed for 10 minutes, after which the color development was stopped by the addition of 100 μ l/well 1.0 M H₃PO₄. The absorbance at 450 nm was read with a reference wavelength of 650 nm (ABS_{450/650}), using a vmax plate reader (Molecular Devices, Palo Alto, CA) controlled with a Macintosh Centris 650 (Apple Computers, Cupertino, CA) and DeltaSoft software (BioMetallics, Inc, Princeton, NJ).

Phosphorylation of Rse.gD was stimulated in a dose dependent fashion and this activity was neutralized by Rse-IgG but not by the control CD4-IgG (Fig. 3D). These data show that a ligand capable of activating Rse is present in FBS.

Example 5

Rse Ligand Characterization

The Rse-L was purified from FBS by ion exchange and Rse affinity chromatography (see Table 2 below).

TABLE 2

Purification of a Rse Ligand from FBS

	Protein (mg)	Units	Yield (%)	Specific Activity (SA) (Units/mg)	Fold Purification
FBS	2800	196	100	0.07	1
QSE	12.8	94	48	7.4	105
Rse-IgG Affinity	0.183	22	11	119	1701

Fetal bovine serum (FBS) was dialyzed (molecular weight cut off 6000 Da) against 50 mM Tris HCl pH 7.5 and sterile filtered (0.22 μ cellulose nitrate, Corning) before loading onto a Q-Sepharose column equilibrated in buffer A, 10 mM Tris HCl, pH 7.5. Buffer B was buffer A with 1M NaCl. The column was eluted with a 1 column volume gradient from 0 to 18% B, then a 10 column volume gradient of 18 to 60% B. Active fractions, eluting near 0.4 M NaCl, were pooled and dialyzed against 50 mM Tris HCl pH 7.5, 5 mM benzamidine. This Q-Sepharose enriched fraction (QSE) was applied to a Rse-IgG affinity column. The column was washed with 50 mM Tris HCl, pH 7.5, 5 mM benzamidine and eluted with 4 M Urea, 0.1M Tris HCl, pH 7.5, 5 mM benzamidine. The eluate was concentrated and dialyzed by centrifugal ultrafiltration (Centricon 10). Rse-IgG columns were prepared using 2 mg of Rse-IgG per ml Emphase resin according to the supplier's instructions (Pierce). The quantities tabulated are relative to 100 ml of FBS starting material. One unit of binding activity is defined as the amount present in 1 ml of a sample having an EC₅₀ of 1% v/v in the *in vitro* binding activation assay described in Example 2 above.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the affinity-purified Rse-L preparation showed a broad band centered at 60 kDa with unreduced samples which resolved into several closely spaced bands from 65 to 68kDa upon reduction. Fractions were heated for 10 min at 90°C in sample buffer, resolved on a 4-20% SDS polyacrylamide gel (Novex) and visualized by silver staining. The eluate of Rse-IgG affinity purified Rse-L was reduced with 25 mM DTT prior to electrophoresis.

The Rse-L preparation was separated by SDS-PAGE under reducing conditions, electroblotted and sequenced. Electroblothing onto Millipore Immobilon PSQ membranes was carried out for 1 h at 250 mA constant current in a Biorad transblot transfer cell as described (P. Matsudaira, *J. Biol. Chem.*, 262:10035 [1987]). The membrane was stained with 0.1% Coomassie Blue R-250 in 50% methanol for 30 sec, destained with 10% acetic acid in 50% methanol for 2 to 3 min, thoroughly washed with distilled water, dried, and stored at 4° C. Automated protein sequencing was performed on models 473A and 490A Applied Biosystems Sequencers equipped with on-line PTH analyzers. Peaks were integrated with Justice Innovation software using Nelson Analytical 760 interfaces. Sequence interpretation was performed as described (Henzel *et al.* *J. Chromatogr.* 404:41 [1987]).

The preparation gave an amino-terminal sequence of XQVLIRRXRANTL [SEQ ID NO: 8], corresponding to that of bovine protein S. Protein S sequences were obtained from several independent preparations of Rse-L. After SDS-PAGE, some preparations were characterized by the presence of a 14 kDa species having an N-terminal sequence of ANTLL [SEQ ID NO: 9], as previously reported for bovine protein S, along with 60-70 kDa species with sequences corresponding to cleavage within the thrombin sensitive loop region of bovine protein S. After CnBr cleavage of the sequencing filter >99% of all identifiable residues were accounted for by a mixture of protein S CnBr fragments. Furthermore, Rse-L activity could not be separated from protein S by anion exchange chromatography in the presence of Ca²⁺, cation exchange chromatography, hydrophobic interaction chromatography, Blue Sepharose chromatography, or nondenaturing gel electrophoresis. The Rse-L activity present in FBS and purified fractions could be neutralized by protein S polyclonal antisera.

Human serum or recombinant human protein S expressed in 293 cells showed low activity in either the KIRA or Rse-IgG binding assays. Human serum was obtained from Pierce and from local blood banks. Human protein S (Calbiochem, Enzyme Research Labs, or Celsus labs) had an EC₅₀ of >250 nM in the membrane binding assay. In comparison, the purified bovine protein S had an EC₅₀ of 1.2 nM in this assay. In the KIRA assay, concentrations as high as 150 nM human protein S resulted in low phosphorylation of Rse. Human protein S cDNAs were obtained by PCR using 1 µg of human fetal liver cDNA (Clontech) as template with Pfu DNA polymerase (Stratagene) as described in Mark *et al.* (1992), *supra*. Human protein S was expressed in 293 cells grown in the presence of 2 µg/ml Vitamin K exactly as described below for human gas6, and expression was verified by metabolic labeling of cultures and/or by western blotting with a polyclonal anti-protein S antiserum. Purified human protein S bound ¹²⁵I-Rse-IgG, but with ~200 fold lower affinity than purified bovine protein S.

It was hypothesized that a homologue of protein S might be more effective. A search of the GENBANK data base revealed substantial similarity (44% amino acid identity, and a similar domain structure) between the amino acid sequence of human protein S and the predicted product of human growth arrest specific gene 6 (gas6) (Manfioletti *et al.*, *supra*).

Example 6

Recombinant Production of gas6

It was determined whether human gas6 was a ligand for Rse. Gas6 cDNA clones were obtained by polymerase chain reaction cloning from reverse transcribed human brain cDNA. The full-length human gas6 clone was constructed by linking together cDNAs encoding amino acids 1-318 and 319-678. Gas6 cDNAs were obtained by PCR using 1 µg of human fetal brain cDNA (Clontech) as template with Pfu DNA polymerase as described (Mark *et al.*, J. Biol. Chem. 267:26166 [1992]). Forward and reverse primers designed to obtain the 5' and 3' portions of hgas6 were:

(5'-GATATCGATCCATGCCCCCTTCGCTCTC [SEQ ID NO:10];

10 5'-CATGGATCCTACCGGAAGTCAAACCTCAGCTA [SEQ ID NO: 11]) and

(5'-GATATCGATGAGTGTGAAGTCTTGTAC [SEQ ID NO: 12];

5'-GTCGGATCCGACAGAGACTGAGAAGCC [SEQ ID NO: 13]), respectively.

Human fetal kidney 293 cells were transiently transfected as described in Mark *et al.*, J. Biol. Chem. 267:26166 (1992). After a 4 h incubation, the media was replaced with growth media plus antibiotics and 2 µg/ml Vitamin K. Conditions for metabolic labeling with ³⁵S-Cys and ³⁵S-Met were as described in Mark *et al.* For precipitation with IgG-fusion proteins, radiolabeled supernatants were first precleared with pansorbin (Calbiochem) for 30 min at room temperature, then incubated with 10 µg of the IgG fusion protein for 4 h at 4°C. Fusion proteins were precipitated with 20 µl of pansorbin, the complexes were collected by centrifugation at 14,000 x g for 1 min, and then washed 3 times with PBS containing 0.1% Triton-X 100. Precipitates were analyzed by SDS-PAGE under reducing conditions (Capon *et al.*, Nature 337:525 [1989]). Radioactivity in the dried gel was analyzed with a Fuji phosphorimager.

Conditioned media from cells metabolically labeled after transfection with a gas6 expression vector contained a 70 kDa protein that could be selectively precipitated by the Rse-IgG fusion protein but not by the control fusion protein CD4-IgG. Conditioned media from unlabeled transfections enhanced binding of ¹²⁵I-Rse-IgG to membranes, and induced phosphorylation of Rse receptor expressed in CHO cells. These data indicated that recombinant human gas6 binds to and activates human Rse receptor.

Recombinant gas6 was purified from conditioned media by affinity chromatography. Human fetal kidney 293 cells were transiently transfected as described in Mark *et al.* (1992), *supra*. After a 4 h incubation, the media was replaced with serum free growth media plus antibiotics and 2 µg/ml Vitamin K. Conditioned media were collected at two and 4 days following transfection. The conditioned media of the transfected cells, but not those of either nontransfected or mock transfected 293 cells, activated binding of ¹²⁵I-Rse-IgG. A liter of pooled conditioned media was clarified by centrifugation, diluted with 1 volume of buffer A (50 mM TrisHCl, pH 7.5, 0.1% CHAPS, 5 mM benzamidine), and applied to a 6 ml Resource Q column (Pharmacia) previously equilibrated with buffer A. The column was eluted with a 12 column volume gradient of 0 to 0.4 M NaCl in

buffer A. The active fractions were pooled and diluted with 1 volume buffer A and applied to a Rse-IgG affinity column that was washed and developed as described (see Example 5 above).

The identity of recombinant gas6 was verified by amino terminal sequence. The sequence of the recombinant material begins with the sequence "AFQVFEEA" [SEQ ID NO: 14]. The signal from the glutamic acid residues in this sequence was weak, consistent with γ carboxylation.

A well known characteristic of Gla containing proteins is their coprecipitation with insoluble barium salts (Dahlbeck, *Biochem J.* 209:837 [1983]; Discipio and Davie, *Biochemistry* 18:899 [1979]). An assay based on this property allowed us to analyze the binding of purified gas6 to 125 I-Rse-IgG in the absence of cell membranes. Samples containing various dilutions of Rse-L in 25 mM HEPES pH 7.2, 0.1% BSA and 0.05% Tween-20 were combined and mixed with 125 I-Rse-IgG diluted in the same buffer in a total volume of 100-120 ml. After a 45 min incubation at room temperature, 1 ml of a freshly prepared ice cold suspension of BaCl_2 (10 mM) in phosphate buffered saline was added to each tube and precipitable radioactivity was collected by centrifugation and aspiration of the supernatant fluid. The dissociation constant for Rse-IgG and gas6 measured in this assay was 0.46 nM (Fig. 5).

Purified gas6 stimulated phosphorylation of Rse in a dose dependent fashion. A time course experiment showed that phosphorylation of Rse was induced within two minutes after addition of purified gas6. Activation of Rse phosphorylation by gas6 was neutralized by Rse-IgG but not by CD4-IgG.

Example 7

Gas6 Expression and Characterization

Gas6 and Rse receptor expression in adult human brain tissues was investigated. A blot containing 2 μ g of polyadenylated RNA from human brain tissues (Clontech) was hybridized with random-primed labeled probes corresponding to amino acids 1- 420 of Rse or to amino acids 358-605 of gas6. The tissues were amygdala, caudate nucleus, corpus callosum, hippocampus, hypothalamus, substantia nigra, subthalamic nucleus, and thalamus.

Consistent with the hypothesis that gas6 might be a ligand for Rse, it was found that gas6 and Rse mRNA are co-expressed in each of these adult human brain tissues.

Astrocytes have been reported to synthesize neurotrophic factors that support the growth and survival of neurons. Moretto *et al.*, *J. Neuropath & Exp Neuro.* 53:78 (1994) and Lin *et al.* *Science* 260:1130 (1993). It was determined whether cultured rat astrocytes also synthesize a ligand for Rse. A northern blot was prepared which contained 1 μ g pf polyadenylated RNA from postnatal day 1 astrocytes or hippocampal neurons prepared from E18 rat embryos. Astrocytes were prepared as described (Banker and Goslin, *Culturing Nerve Cells* [MIT Press, Cambridge, 1991], pp 260-261) and then cultured in serum-free media for 1 day, 3 days, or 5 days. Hippocampal neurons were cultured in serum free defined media for 0 days, 3 days or 4 days. The blot was

hybridized with a ^{32}P -labeled probe corresponding to amino acids 1-460 of murine gas6. The blot was stripped then hybridized with a ^{32}P -labeled actin probe to confirm the integrity of the RNA samples.

Gas6 mRNA was detected in cultured type 1 astrocytes prepared from postnatal day 1 rats, but could not be detected in E18 hippocampal neurons.

- 5 Expression data for gas6 and Rse obtained herein and elsewhere is summarized in the following table.

TABLE 3

Expression of Gas6 and Rse in Primary Cell Culture and Cell Lines

Cell Culture	Gas6	Rse
Neuronal System		
Astrocyte	+	+
Hippocampus neurons	-	Schulz <i>et al.</i> , <i>supra</i>
P45 Schwann's	-	+
Neuroglioma		
G28	+	-
G59	+	-
G111	+	-
Glioblastoma, astrocytoma		
U87MG	-	-
U373MG	+	-
Hematopoietic System		
JM, a CML line	n/a	+
CMK11-5	n/a	+
Jurkat	n/a	+
NIH3T3	+	+
293	+	-

The ability of cultured rat astrocytes to also synthesize a ligand for the Rse receptor was investigated. See figure legends for Figs. 6A-6C. Astrocyte conditioned media contained a factor which bound ^{125}I -Rse-IgG

(Fig. 6A) and stimulated tyrosine phosphorylation of Rse (Fig. 6B). This activity was neutralized by Rse-IgG but not CD4-IgG (Fig. 6C).

Example 8

Gas6 Variants

To further characterize the interactions of gas6 with cell membranes and with Rse, a series of N-terminal deletion variants containing an epitope tag were constructed.

The coding sequences for the gD signal sequence and epitope tag (Mark *et al.*, [1992] *supra*) were fused via an XhoI site that was added by PCR to coding sequences immediately before the first amino acid of mature gas6 (gD.gas6; forward primer 5'-AGCTGCTCGAGGCGCTGTTGCCGCGC [SEQ ID NO: 15]) or protein S (gD.protein S; forward primer 5'-AGCTGCTCGAGGCAAAATCTTTACTTGAA [SEQ ID NO: 16], or amino acids 118 (gD.gas6.118-C; forward primer 5'-AGCTGCTCGAGGACCATGTCACGCCCAACC [SEQ ID NO: 17]) and 279 (gD.gas6.279-C; forward primers 5'-GCTGCTCGAGGACATCTTGCCGTGCGTG [SEQ ID NO: 18]) of gas6. The reverse primer for gD.gas6 and gD.gas6.118-C was 5'-CATGGA TCCTACCGGAAGTCAAACCTAGCTA [SEQ ID NO: 11]. The reverse primers for gD.gas6.279-C and gD.protein S were 5'-GTCGATCCGACAGAGACTGAGAAGCC [SEQ ID NO: 13] and 5'-CATTCATTTATGTCAAATTCA [SEQ ID NO: 19], respectively. Gas6.gD was constructed by fusing the coding sequences of gas6 to the C-terminal gD tag used for Rse.gD through an NheI site which was added by PCR using the primers 5'-ATGGAGATCAAGGTCTG [SEQ ID NO: 20] and 5'-CATCTTGAGGCTAGCGGCTGCGGCGGCTCCAC [SEQ ID NO: 21]. The polypeptides were expressed in 293 cells using the procedure essentially as described for full length gas6 in Example 6.

gD.gas6.118-C and gD.gas6.279-C, containing the EGF repeats and tandem G domains within the D domain, or just the G domains, respectively, were precipitated by Rse-IgG (Fig. 7) from cell culture supernatants. Human protein S was not precipitated in this assay which is consistent with the above observations that human protein S binds Rse with a lower affinity than human gas6. These derivatives of gas6 that were truncated for the Gla domain (*i.e.* the A domain) also fail to associate with membranes in a Ca^{2+} fashion.

This data shows that gas6 binds to Rse through the G domains, that the membrane binding and Rse-binding activities are separable, and suggests that the Gla domain is required for Ca^{2+} dependent association with cell membranes.

The gas6 variants described in this example were functionally active. In particular, gD.gas6.118-C and gD.gas6.279-C activated Rse phosphorylation in the KIRA assay described in Example 4 as effectively as full-length gD-tagged gas6 (see Fig. 7).

Example 9

Schwann Cell Proliferation Assay

Rse mRNA, but not gas6 mRNA was also detected in the rat Schwann cell line rhesC, which is derived from rat E14 dorsal root ganglia. Addition of purified gas6 to these cells resulted in a dose dependent increase in cell number (50% increase at 48 hours) with an EC_{50} of ~ 0.3 nM (Fig. 8). Gas6 treatment also altered the morphology of these cells; untreated cells were multipolar with numerous branched processes whereas gas6 treated cells became spindle-shaped with two major smooth processes and aligned themselves in a parallel array. It was also demonstrated that gas6-induced proliferation was neutralized by Rse-IgG but not CD4-IgG. See Fig. 9.

Using Rse- and Axl- specific antibodies, Rse and Axl receptor tyrosine kinases were also detected in human Schwann cells. The ability of gas6 to enhance the proliferation of human Schwann cells was determined.

Peripheral nerve tissues were obtained at the University of Miami School of Medicine, with appropriate patient consent, as previously described (Levi *et al.*, *J. Neuroscience* 15(2):1329-1340 [1995]). Pieces of peripheral nerve fibers were placed in Belzer's UW solution and shipped to California. Upon receipt, the nerve fibers were washed with fresh F12/DME (1:1) and incubated with 1% collagenase/dispase solution (Boehringer) at 37°C for 30 minutes. Then, the tissue was gently washed 3x by transferring the tissue to fresh tissue culture medium. The fibers were plated in 100 mm petri dishes in serum free medium supplemented with the following formula for rat Schwann cells: F12/DME (1:1) supplemented with insulin (10 μ g/ml), transferrin (10 μ g/ml), α -tocopherol (5 μ g/ml), recombinant human heregulin- β 1₁₇₇₋₂₄₄ produced as described in Holmes *et al.*, *Science*, 256: 1205-1210 (1992) (10 nmole/L), forskolin (5 μ molar), progesterone (3×10^{-4} molar), and bovine pituitary extract (BPE) (3 μ l/ml). The Schwann cells were cultured in suspension for 48 hours to allow partial demyelination. The nerve fibers were then pooled by centrifugation at 1000rpm for 5 minutes and resuspended and dispersed by gentle pipetting.

The dispersed Schwann cells were replated on laminin (Gibco BRL) coated tissue culture 48 well multiplates at 8×10^3 cells/well in defined medium with the addition of aprotinin (25 μ g/ml) and 50 μ L/ml chemically defined lipids (Sigma Cat# 11905-015; Gibco BRL). These cultures were designated the "primary culture". Medium was changed every 5 days. Confluent cultures of pure Schwann cells could be obtained within 2 weeks. At the first and second passage, cells were removed from the plate using collagenase/dispase (Boehringer Mannheim), washed with medium containing 3% BSA, and plated as described. The media used were "6F" medium: F12/DME (1:1) supplemented with insulin (10 μ g/ml), transferrin (10 μ g/ml), α -tocopherol (5 μ g/ml), progesterone (3×10^{-4} molar), aprotinin (25 μ g/ml) and chemically defined lipids (Sigma Cat# 11905-015). "8F" medium contains the supplements of 6F medium as well as recombinant human heregulin- β 1₁₇₇₋₂₄₄ (10 nmole/L) and forskolin (5 μ M). The effect of gas6 on Schwann cell survival and proliferation was studied by adding gas6 to either of these culture mediums.

Gas6 stimulates human Schwann cell growth in a dose dependent manner (Fig. 12A) with a significant effect seen at 1 ng/ml (14pM) and maximal effect with doses over 10 ng/ml. Gas6 alone produces a significant increase in Schwann cell number compared to control medium. In the presence of the cAMP activator, forskolin, the increase in total cell number with gas6 is more pronounced. A synergistic effect is also observed between gas6 and heregulin. Gas6 increased both cell number and thymidine incorporation even in the presence of preferred concentrations of both forskolin and heregulin (Fig. 12B).

In the presence of the preferred concentrations of both heregulin and forskolin, other growth factors previously reported to stimulate Schwann cell growth had no effect (PDGF, FGF- β) or reduce cell number (IL-1 α and TGF- β 1) (Fig. 12C). Addition of human or bovine protein S at 10ng-5 μ g did not increase Schwann cell number after 5 days of culture. In contrast, gas6 at 30 μ g/ml maximally increased the cell number. The combination of gas6 with forskolin and heregulin results in maximal cell growth over a 5 day period comparable to that seen in the combination of 6F + forskolin + heregulin + 5% FBS.

Gas6 has a marked effect on cell morphology as determined by viewing phase contrast micrographs of human Schwann cells grown in 6F+heregulin; 6F+heregulin+gas6; 8F+gas6; and 8F+10% fetal bovine serum. Micrographs were taken after 96 hours of culture. The Schwann cell grown in the presence of gas6 have processes which are much longer than those seen in cells grown in the presence of heregulin or heregulin with forskolin. Mitotic figures may also clearly be seen in the 8F + gas6 cultures even in those cells with the fully developed Schwann cell spindle-shaped morphology. The addition of serum to the 8F cultures alters cell morphology causing the cells to flatten, spread and eventually become vacuolated.

Cells were stained by immunofluorescence for the Schwann cell markers GFAP and S100 protein. Briefly, passage 4 human Schwann cells grown in 8F+gas6 were cultured for 24 hours on laminin coated Chamber slides and fixed in 10% formalin in PBS. Fixed cells were blocked with 10% goat serum and incubated with rabbit anti-GFAP (ICN) and anti-S-100 protein (ICN) at dilutions recommended by the distributor. Specific binding of the primary antibody was stained with goat anti-rabbit IgG (Fab')₂-FITC conjugates. Cells were counter-stained with DNA dye propidium iodide. Negative controls were run on WI-38 cells which stained negative. Cells grown showed 100% immunofluorescent staining for the Schwann cell markers GFAP and S100 protein after 4 subcultures.

The ability of gas6 to stimulate human Schwann cell proliferation through the Axl-Rse family of tyrosine kinase receptors was investigated. Human Schwann cells were stimulated with 0, 0.01, 0.1 or 1 μ g/ml of human gas6 (hgas6) for 15 min at 37°C incubator. Cell lysates were prepared and immunoprecipitated with rabbit anti-hRseFc fusion protein antibody and rabbit anti-hAxl antibody. Tyrosine phosphorylation of hRse and hAxl receptor was detected with 4G10 anti-phosphorylation antibody. 10⁶ human Schwann cells were grown to near confluence in defined media (8F+gas6) and changed to 6F 24 hours before experiment. Cells were treated with purified recombinant hgas6 for 15 min in 37°C incubator and lysed on ice with 1 ml of lysis buffer (20 mM HEPES, pH7.4, 135 mM NaCl, 50 mM NaF, 1 mM sodium vanadate and 1 mM sodium molybdate,

- 2 mM EDTA and 2 mM EGTA, 10% glycerol, 1%NP40, 1 μ M okadaic acid, 1 mM PMSF and 1 mM AEBF). Cell lysates were clarified by centrifuging at 14000xg 4°C for 10 min. Immunoprecipitations were performed using 1 μ g of rabbit anti-hRseFc fusion protein antibody or 2 μ l of rabbit anti-hAxl antiserum raised against the 10 amino acids at the COOH-terminal of hAxl at 4°C for 2 hrs. Immunocomplex were collected with 10 μ l of
- 5 Protein A Sepharose CL-4B beads. Proteins were separated on Novex 4-12% gradient gel and transferred onto nitrocellulose membrane. Anti-phosphotyrosine immunoblots were performed using 4G10 mouse anti-phosphotyrosine antibody (UBI), goat anti-mouse horseradish peroxidase conjugate and ECL developing kit (Amersham). Addition of human gas6 to human Schwann cells causes autophosphorylation of both Axl and Rse receptors on tyrosine residue(s). Activation of Axl and Rse could be detected at 1.4-14 μ M gas6. Such
- 10 phosphorylation of Axl and Rse is not observed in cultures stimulated with heregulin. Gas6 expression in cultured rat Schwann cells was not detected by northern blot. Furthermore, gas6 activity in rat Schwann cell conditioned medium was not seen. Without being bound by any one theory, it is possible that gas6 is produced by growing axons, or by nearby fibroblast cells (from which gas6 was initially cloned). This activation of Axl-Rse receptors on Schwann cells by gas6 is highly specific, since growth factors known to act via other tyrosine
- 15 kinase receptors, such as PDGF and FGF, do not increase human Schwann cell proliferation under these defined conditions. The Schwann cell growth factors, GGF/heregulin, acting independently through the *erbB* receptor family, synergize with gas6 in this study.

- It is beneficial to have populations of mammalian Schwann cells (preferably human Schwann cells) for use as cellular prostheses for transplantation into areas of damaged spinal cord in an effort to influence
- 20 regeneration of interrupted central axons, for assisting in the repair of peripheral nerve injuries and as alternatives to multiple autografts. See Levi *et al.*, *J. Neuroscience* 14(3):1309-1319 (1994). The use of cell culture techniques to obtain an abundant source of autologous graft material from a small biopsy has already met with clinical success in providing human epidermal cells to cover extensive burns (Gallico *et al.*, *N. Eng. J. Med.*, 311:338-451 [1984]). Furthermore, it has been shown that Schwann cells from human xenografts are capable
- 25 of myelinating regenerating peripheral axons from mice which have been immunosuppressed (Aguayo *et al.*, *Nature* 268:753-755 [1977], and Aguayo *et al.*, *Soc. Neurosci. Symp.* 4:361-383 [1979]). Accordingly, it is expected that the above approach will meet with success in mammals, including humans.

Example 10

30 Gas6 Immunoadhesin

gD.gas6.279-C.IgG was constructed by fusing the coding sequences of gD.gas6.279-C (see Example 8) to amino acids 216-443 of human IgGy1 through a BstEII linker (adding amino acids Val and Thr). The linker was added to gD.gas6.279-C sequences by PCR using the primers 5'-ATGGAGATCAAGGTCTG [SEQ ID NO: 20] and 5'-GTCCGGTACCGCTGCTGCGGGCTCCAC [SEQ ID NO: 22].

The gas6 immunoadhesin thus formed was subjected to the KIRA assay described in Example 4 above. Briefly, different dilutions of conditioned media from cells transiently expressing gD.gas6.279-C.IgG were tested in the KIRA assay. The starting material had a concentration of gD.gas6.279-C.IgG of ~230 ng/ml. The EC₅₀ for activation was approximately 0.4 nM. See Fig. 10. Activity was not observed in conditioned media from transiently transfected control cell lines.

Example 11

Activation of Rse by Non-γ Carboxylated Gas6

Media (700 ml) conditioned for 3 days by 293 cells transfected with human gas6 (hgas6.17) was dialyzed against 2 x 8 L of 50 mM Tris-HCl pH 7.5, 5 mM benzamidine (buffer A). The dialyzate was adjusted to 0.1 % CHAPS, and loaded on a 6 ml Resource-Q column (Pharmacia) at 10 ml/minute. The column was washed with buffer A, and eluted with a 70 ml linear gradient of 0 to 0.4 M NaCl in buffer A, at a flow rate of 1.0 ml/min, collecting fractions of 2.0 ml.

The fractions were analyzed for their ability to bind and activate Rse using the barium chloride binding method described in Example 6 and the KIRA assay described in Example 4. The barium chloride assay can only detect binding of Gla containing Rse ligands, while the KIRA assay is sensitive to all Rse activators. Binding activity was centered at fraction 31, while KIRA showed an additional earlier eluting peak centered at fraction 24.

Aliquots (10 μl) of fractions 20 to 44 were analyzed on 8% acrylamide (Novex) SDS gels, and proteins visualized by silver staining. In these fractions a 75 kD band comigrated with standard hgas6. Integrated intensities of the 75 kD band were measured by laser scanning (Molecular Dynamics) and image analysis (NIH Image). Peak intensities were found in fractions 24 and 31, corresponded to the 2 regions of KIRA activity. The amount of hgas6 in each fraction was estimated from the staining intensity of a known quantity (0.34 μg) of a standard preparation of hgas6 run on the same gel, assuming a linear relationship between staining intensity and protein load.

Sequence analysis of the 75 kD bands from fractions 24 and 31 was performed after SDS-PAGE and electrophoretic transfer to PVDF membranes. The amino terminal sequence of both bands was unambiguously identified as that of hgas6 (AFQVF), but the two could be differentiated by the presence or absence of modified glutamic acid residues in later cycles. The sequence from fraction 31 lacked a signal from glutamic acid in cycles 6,7,14, and 16, consistent with a γ-carboxyl modification of these residues. The sequence from fraction 24 was consistent with unmodified glutamic acid at all these positions.

Both sequence analysis and binding behavior of the early eluting form of hgas6 are consistent with its identification as an unmodified form of hgas6, lacking the characteristic γ-carboxyl modification of glutamic acid. This second discovered form of recombinant hgas6 appears to be more active than the first described Gla containing form. The specific activity of the two forms was calculated from the KIRA data in Fig. 11 and from

the densitometric quantitation of hgas6. The specific activity of fraction 31 (form 1) is 1170 KIRA units/mg P, while that of fraction 24 (form 2) is 3158. This indicates that form 2, lacking the Glu modification, is more potent than the Glu containing form 1 in activating Rse.

Example 12

Activation of Mer Receptor by gas6

A cDNA encoding human Mer was obtained by screening a human testis cDNA library prepared in lambda DR2 (Clontech, Palo Alto CA) with oligonucleotide probes:

5'-GAAATTACAGATCCGCAGCCCGGGATGCGGCGGCCCGCTGCCGCTGC [SEQ ID NO: 23],
5'-CCTTGGATTCTAGCAAGCAGCACTGAAGGAGCCCCATCAGTAGCACCTTT [SEQ ID NO: 24], and
5'-TCTTAAAAATTAAGCTTCAGCTGCTCCTTGATATTAACCTTTGTACAGAGT-3' [SEQ ID NO: 25].

Positive phage were purified and insert sizes were determined. Three overlapping clones hMer.c14, hMer.c125, and hMer.c16 (corresponding to nt 1-561, nt 223-2025, and nt 1902-3608 of published sequence respectively) were combined to make a cDNA encoding the entire open reading frame of human Mer.

Mer-Fc was constructed by fusing the sequence encoding amino acids 1-499 of human Mer to amino acids 216-443 of human IgGy1 through a NarI-BstEII linker (5'-GCGCCTGGCAACGCG-3' [SEQ ID NO: 26], 5'-GTGACCGCGTGGCCAG-3' [SEQ ID NO: 27]) (adding amino acids glycine and histidine). Human embryonic kidney (HEK) 293 cells expressing Mer-Fc were screened using a human Fc-specific ELISA. Mer-Fc was purified on a protein A-sepharose column (Pharmacia).

Epitope tagged gD.Mer was constructed by fusing the coding sequences for amino acids 1-53 of herpes simplex virus type 1 (HSV 1) glycoprotein D (gD) to the sequences encoding amino acids 36-961 of human Mer. See Example 3 above. Oligos (5'-CGAATTCCTCGAGCCGGACCTTTCCAGGGAGC-3' [SEQ ID NO: 28], and

5'-CCAACCTGTGTGTTGAAGGCAAGAGGCGG-3' [SEQ ID NO: 29]) were used to add a XhoI site to the human Mer cDNA by PCR. The gD.Mer cDNA was inserted into a CMV-based expression vector and HEK 293 cells were transfected, selected and screened by western blotting and fluorescent activated cell sorting as described above in Example 3.

Recombinant human gas6 and Protein S were expressed as described in Godowski *et al. Cell* 82:355-358 (1995). Expression was verified by metabolic labeling of cultures with ³⁵S-Cys and ³⁵S-Met (Mark *et al., J. Biol. Chem.* 267:26166 [1992]) and/or by western blotting with rabbit polyclonal anti-gas6 or anti-Protein S antiserum (Sigma). N-terminal tagged versions of Protein S or gas6 were constructed as described in Godowski *et al., (1995) supra*.

Methods to detect Rse phosphorylation using the KIRA ELISA and Western analysis using anti-phosphotyrosine antibodies have been described above in Examples 3 and 4. For neutralization experiments, potential ligand sources were treated at room temperature for 30 min with the indicated Fc fusion protein prior

to addition to cells. To analyze the ability of potential ligands to induce phosphorylation of gD.Mer, 500,000 cells were seeded on a 60 mm dish in the presence of serum for 6 h. The cells were then washed twice in PBS and serum-starved for 16 h. Potential ligands were added to the cells and gD.Mer was immunoprecipitated from lysates using 5B6, analyzed by SDS-PAGE under reducing conditions, and blotted with anti-phosphotyrosine antibody (4G10).

For binding to Rse-Fc, Axl-Fc, and Mer-Fc, conditioned media containing 5-10 nM gas6 or protein S was precleared with protein A-Sepharose (Calbiochem) for 30 min at room temperature, then incubated with 5 μ g of the receptor Fc fusion protein for 4 h at 4°C. Fusion proteins were immunoprecipitated with 20 μ l of protein A sepharose and the complexes were collected by centrifugation at 14,000 x g for 1 min, and then washed 3 times with PBS containing 0.1% Triton X-100. Precipitates were analyzed by SDS-PAGE under reducing conditions. Western blots of the SDS-PAGE gels were probed with antibody 5B6.

Protein interaction analysis using BIAcore™ instruments were performed with Mer-Fc coupled to BIAcore CM5 sensor chips using purified gas6 and Protein S. For neutralization experiments, 5 μ g of either Mer-Fc or CD4-Fc was mixed with gas6 for thirty minutes at room temperature prior to injection over the chip. Sensorgrams were analyzed with BIA evaluation 2.1 software from Pharmacia Biosensor AB. Apparent dissociation rate constants (k_d) and association rate constants (k_a) were obtained by evaluating the sensorgram with A+B=AB type I fitting. Equilibrium dissociation constant K_d was calculated as k_d/k_a .

Results

The ability of Mer-Fc to neutralize gas6-induced phosphorylation of Rse in the ELISA based KIRA assay was investigated. Activation of Rse by gas6 was blocked by Rse-Fc and Mer-Fc in a dose-responsive fashion (Fig. 14). Neutralization was specific to Rse-Fc and Mer-Fc, because the control protein CD4-Fc did not inhibit in this assay. Rse-Fc was somewhat more potent than Mer-Fc in neutralizing gas6. These data suggest that Mer-Fc, like Rse-Fc, blocks activation of Rse by binding to gas6.

The ability of Axl-Fc, Rse-Fc and Mer-Fc to bind directly to gas6 was determined using a coprecipitation assay. The ability of these receptors to bind Protein S was also determined. To allow a more quantitative comparison of their binding properties, versions of gas6 and Protein S that contained epitope tags were utilized. Conditioned media from cells expressing tagged gas6 or Protein S was incubated with each of the receptor fusion proteins. The Fc fusion proteins, and proteins bound to them, were recovered with protein A and washed extensively. Tagged proteins that bound to the Fc-fusion proteins were revealed by Western blotting and detection with an antibody directed against the epitope tag. Both Axl-Fc and Rse-Fc bound tagged gas6 but not Protein S. An identical result was observed with Mer-Fc. The binding was specific in that neither gas6 nor Protein S bound the control CD4-Fc.

The kinetics of the interaction of gas6 with the extracellular domains of Mer and Rse was compared using a BIAcore instrument. Mer-Fc was immobilized on a biosensor chip, and various concentrations of gas6

were passed over the chip. A representative sensorgram from such an experiment is shown in Fig. 15A. No binding was observed to Protein S and binding of gas6 to Mer-Fc immobilized on the chip was blocked by soluble Mer-Fc, but not CD4-Fc (Fig. 15). The dissociation constant (K_d) for the interaction of gas6 with Mer-Fc was 6 nM. The K_d for binding of gas6 to Rse-Fc was 4.2 nM.

5 HEK 293 cells were transfected with an expression vector encoding a version of Mer containing an amino-terminal epitope tag. A candidate cell line (termed 293.gD.Mer) expressing gD.Mer was identified by fluorescence-activated cell sorting using 5B6, a monoclonal antibody that recognizes the epitope tag. Incubation of these cells with antibody 5B6 resulted in rapid phosphorylation of novel proteins of approximately 180 and 200 kDa that were absent in control, untransfected HEK 293 cells. Receptor phosphorylation was not observed
10 when the cells were incubated with a control antibody. These results indicate that Mer encodes a functional tyrosine kinase.

The ability of human gas6 and Protein S to activate the kinase activity of Mer was investigated. Mer did not become phosphorylated when these cells were treated with Protein S. However, receptor phosphorylation was detected in cells treated with gas6. A time course experiment demonstrated that Mer was phosphorylated
15 within minutes of addition of gas6 and phosphorylation could be detected after stimulation of cells with gas6 at concentrations of 1-3nM.

The ability of gas6 to induce the phosphorylation of Mer expressed endogenously was determined. Mer mRNA was detected in the monocytic leukemia cell line THP-1 by reverse transcription PCR. Using a polyclonal antibody directed against the extracellular domain of Mer, expression of Mer on the surface of THP-1 cells was
20 confirmed. Treatment of these cells with gas6 induced the rapid phosphorylation of a 180 kDa protein that was immunoprecipitated with antibodies to Mer. These observations show that gas6 is a functional ligand for Mer.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Genentech, Inc.
Chen, Jian
5 Godowski, Paul J.
Hammonds, R. Glenn
Mark, Melanie
Mather, Jennie P.
Li, Ronghao
- 10 (ii) TITLE OF INVENTION: RECEPTOR ACTIVATION BY GAS6
- (iii) NUMBER OF SEQUENCES: 29
- (iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 460 Point San Bruno Blvd
15 (C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080
- (v) COMPUTER READABLE FORM:
20 (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)
- (vi) CURRENT APPLICATION DATA:
25 (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
30 (A) APPLICATION NUMBER: 08/402253
(B) FILING DATE: 3/10/95
- (vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/438861
(B) FILING DATE: 5/10/95
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40 (A) TELEPHONE: 415/225-1994
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 673 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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	Leu	Leu	Leu	Leu	Leu	Ala	Ser	Glu	Ser	Ser	His	Thr	Val	Leu	Leu	
10						20					25				30	
	Arg	Ala	Arg	Glu	Ala	Ala	Gln	Phe	Leu	Arg	Pro	Arg	Gln	Arg	Arg	
						35					40				45	
	Ala	Tyr	Gln	Val	Phe	Glu	Glu	Ala	Lys	Gln	Gly	His	Leu	Glu	Arg	
						50					55				60	
15	Glu	Cys	Val	Glu	Glu	Val	Cys	Ser	Lys	Glu	Glu	Ala	Arg	Glu	Val	
						65					70				75	
	Phe	Glu	Asn	Asp	Pro	Glu	Thr	Glu	Tyr	Phe	Tyr	Pro	Arg	Tyr	Gln	
						80					85				90	
	Glu	Cys	Met	Arg	Lys	Tyr	Gly	Arg	Pro	Glu	Glu	Lys	Asn	Pro	Asp	
20						95					100				105	
	Phe	Ala	Lys	Cys	Val	Gln	Asn	Leu	Pro	Asp	Gln	Cys	Thr	Pro	Asn	
						110					115				120	
	Pro	Cys	Asp	Lys	Lys	Gly	Thr	His	Ile	Cys	Gln	Asp	Leu	Met	Gly	
						125					130				135	
25	Asn	Phe	Phe	Cys	Val	Cys	Thr	Asp	Gly	Trp	Gly	Gly	Arg	Leu	Cys	
						140					145				150	
	Asp	Lys	Asp	Val	Asn	Glu	Cys	Val	Gln	Lys	Asn	Gly	Gly	Cys	Ser	
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	Gln	Val	Cys	His	Asn	Lys	Pro	Gly	Ser	Phe	Gln	Cys	Ala	Cys	His	
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	Ser	Gly	Phe	Ser	Leu	Ala	Ser	Asp	Gly	Gln	Thr	Cys	Gln	Asp	Ile	
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	Asp	Glu	Cys	Thr	Asp	Ser	Asp	Thr	Cys	Gly	Asp	Ala	Arg	Cys	Lys	
						200					205				210	
35	Asn	Leu	Pro	Gly	Ser	Tyr	Ser	Cys	Leu	Cys	Asp	Glu	Gly	Tyr	Thr	
						215					220				225	

	Tyr	Ser	Ser	Lys	Glu	Lys	Thr	Cys	Gln	Asp	Val	Asp	Glu	Cys	Gln	
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	Gln	Asp	Arg	Cys	Glu	Gln	Thr	Cys	Val	Asn	Ser	Pro	Gly	Ser	Tyr	
					245					250					255	
5	Thr	Cys	His	Cys	Asp	Gly	Arg	Gly	Gly	Leu	Lys	Leu	Ser	Pro	Asp	
					260					265					270	
	Met	Asp	Thr	Cys	Glu	Asp	Ile	Leu	Pro	Cys	Val	Pro	Phe	Ser	Met	
					275					280					285	
10	Ala	Lys	Ser	Val	Lys	Ser	Leu	Tyr	Leu	Gly	Arg	Met	Phe	Ser	Gly	
					290					295					300	
	Thr	Pro	Val	Ile	Arg	Leu	Arg	Phe	Lys	Arg	Leu	Gln	Pro	Thr	Arg	
					305					310					315	
	Leu	Leu	Ala	Glu	Phe	Asp	Phe	Arg	Thr	Phe	Asp	Pro	Glu	Gly	Val	
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					335					340					345	
	Gly	Leu	Arg	Ala	Gly	Arg	Leu	Glu	Leu	Gln	Leu	Arg	Tyr	Asn	Gly	
					350					355					360	
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					365					370					375	
	Trp	Gln	Thr	Ile	Ser	Val	Glu	Glu	Leu	Glu	Arg	Asn	Leu	Val	Ile	
					380					385					390	
	Lys	Val	Asn	Lys	Asp	Ala	Val	Met	Lys	Ile	Ala	Val	Ala	Gly	Glu	
					395					400					405	
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					410					415					420	
	Gly	Gly	Ile	Pro	Phe	Lys	Glu	Ser	Glu	Leu	Val	Gln	Pro	Ile	Asn	
					425					430					435	
30	Pro	Arg	Leu	Asp	Gly	Cys	Met	Arg	Ser	Trp	Asn	Trp	Leu	Asn	Gly	
					440					445					450	
	Glu	Asp	Ser	Ala	Ile	Gln	Glu	Thr	Val	Lys	Ala	Asn	Thr	Lys	Met	
					455					460					465	
	Gln	Cys	Phe	Ser	Val	Thr	Glu	Arg	Gly	Ser	Phe	Phe	Pro	Gly	Asn	
					470					475					480	
35	Gly	Phe	Ala	Thr	Tyr	Arg	Leu	Asn	Tyr	Thr	Arg	Thr	Ser	Leu	Asp	
					485					490					495	

	Val Gly Thr Glu Thr Thr Trp Glu Val Lys Val Val Ala Arg Ile	
	500	510
	Arg Pro Ala Thr Asp Thr Gly Val Leu Ala Leu Val Gly Asp	
	515	525
5	Asp Asp Val Val Ile Ser Val Ala Leu Val Asp Tyr His Ser Thr	
	530	540
	Lys Lys Leu Lys Lys Gln Leu Val Val Leu Ala Val Glu Asp Val	
	545	555
	Ala Leu Ala Leu Met Glu Ile Lys Val Cys Asp Ser Gln Glu His	
10	560	570
	Thr Val Thr Val Ser Leu Arg Glu Gly Glu Ala Thr Leu Glu Val	
	575	585
	Asp Gly Thr Lys Gly Gln Ser Glu Val Ser Thr Ala Gln Leu Gln	
	590	600
15	Glu Arg Leu Asp Thr Leu Lys Thr His Leu Gln Gly Ser Val His	
	605	615
	Thr Tyr Val Gly Gly Leu Pro Glu Val Ser Val Ile Ser Ala Pro	
	620	630
	Val Thr Ala Phe Tyr Arg Gly Cys Met Thr Leu Glu Val Asn Gly	
20	635	645
	Lys Ile Leu Asp Leu Asp Thr Ala Ser Tyr Lys His Ser Asp Ile	
	650	660
	Thr Ser His Ser Cys Pro Pro Val Glu His Ala Thr Pro	
	665	673

25 (2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 678 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Pro Ser Leu Ser Pro Gly Pro Ala Ala Leu Arg Arg Ala	
1	15
Pro Gln Leu Leu Leu Leu Leu Leu Ala Ala Glu Cys Ala Leu Ala	
20	30
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	65	70 75
5	Arg Glu Val Phe Glu Asn Asp Pro Glu Thr Asp Tyr Phe Tyr Pro	
	80	85 90
	Arg Tyr Leu Asp Cys Ile Asn Lys Tyr Gly Ser Pro Tyr Thr Lys	
	95	100 105
10	Asn Ser Gly Phe Ala Thr Cys Val Gln Asn Leu Pro Asp Gln Cys	
	110	115 120
	Thr Pro Asn Pro Cys Asp Arg Lys Gly Thr Gln Ala Cys Gln Asp	
	125	130 135
	Leu Met Gly Asn Phe Phe Cys Leu Cys Lys Ala Gly Trp Gly Gly	
	140	145 150
15	Arg Leu Cys Asp Lys Asp Val Asn Glu Cys Ser Gln Glu Asn Gly	
	155	160 165
	Gly Cys Leu Gln Ile Cys His Asn Lys Pro Gly Ser Phe His Cys	
	170	175 180
20	Ser Cys His Ser Gly Phe Glu Leu Ser Ser Asp Gly Arg Thr Cys	
	185	190 195
	Gln Asp Ile Asp Glu Cys Ala Asp Ser Glu Ala Cys Gly Glu Ala	
	200	205 210
	Arg Cys Lys Asn Leu Pro Gly Ser Tyr Ser Cys Leu Cys Asp Glu	
	215	220 225
25	Gly Phe Ala Tyr Ser Ser Gln Glu Lys Ala Cys Arg Asp Val Asp	
	230	235 240
	Glu Cys Leu Gln Gly Arg Cys Glu Gln Val Cys Val Asn Ser Pro	
	245	250 255
30	Gly Ser Tyr Thr Cys His Cys Asp Gly Arg Gly Gly Leu Lys Leu	
	260	265 270
	Ser Gln Asp Met Asp Thr Cys Glu Asp Ile Leu Pro Cys Val Pro	
	275	280 285
	Phe Ser Val Ala Lys Ser Val Lys Ser Leu Tyr Leu Gly Arg Met	
	290	295 300
35	Phe Ser Gly Thr Pro Val Ile Arg Leu Arg Phe Lys Arg Leu Gln	
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	Glu Gly Ile Leu Leu Phe Ala Gly Gly His Gln Asp Ser Thr Trp	
	335	345
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	350	360
	Tyr Asn Gly Val Gly Arg Val Thr Ser Ser Gly Pro Val Ile Asn	
	365	375
10	His Gly Met Trp Gln Thr Ile Ser Val Glu Glu Leu Ala Arg Asn	
	380	390
	Leu Val Ile Lys Val Asn Arg Asp Ala Val Met Lys Ile Ala Val	
	395	405
	Ala Gly Asp Leu Phe Gln Pro Glu Arg Gly Leu Tyr His Leu Asn	
	410	420
15	Leu Thr Val Gly Gly Ile Pro Phe His Glu Lys Asp Leu Val Gln	
	425	435
	Pro Ile Asn Pro Arg Leu Asp Gly Cys Met Arg Ser Trp Asn Trp	
	440	450
20	Leu Asn Gly Glu Asp Thr Thr Ile Gln Glu Thr Val Lys Val Asn	
	455	465
	Thr Arg Met Gln Cys Phe Ser Val Thr Glu Arg Gly Ser Phe Tyr	
	470	480
	Pro Gly Ser Gly Phe Ala Phe Tyr Ser Leu Asp Tyr Met Arg Thr	
	485	495
25	Pro Leu Asp Val Gly Thr Glu Ser Thr Trp Glu Val Glu Val Val	
	500	510
	Ala His Ile Arg Pro Ala Ala Asp Thr Gly Val Leu Phe Ala Leu	
	515	525
30	Trp Ala Pro Asp Leu Arg Ala Val Pro Leu Ser Val Ala Leu Val	
	530	540
	Asp Tyr His Ser Thr Lys Lys Leu Lys Lys Gln Leu Val Val Leu	
	545	555
	Ala Val Glu His Thr Ala Leu Ala Leu Met Glu Ile Lys Val Cys	
	560	570
35	Asp Gly Gln Glu His Val Val Thr Val Ser Leu Arg Asp Gly Glu	
	575	585

Ala Thr Leu Glu Val Asp Gly Thr Arg Gly Gln Ser Glu Val Ser
 590 595 600

Ala Ala Gln Leu Gln Glu Arg Leu Ala Val Leu Glu Arg His Leu
 605 610 615

5 Arg Ser Pro Val Leu Thr Phe Ala Gly Gly Leu Pro Asp Val Pro
 620 625 630

Val Thr Ser Ala Pro Val Thr Ala Phe Tyr Arg Gly Cys Met Thr
 635 640 645

10 Leu Glu Val Asn Arg Arg Leu Leu Asp Leu Asp Glu Ala Ala Tyr
 650 655 660

Lys His Ser Asp Ile Thr Ala His Ser Cys Pro Pro Val Glu Pro
 665 670 675

Ala Ala Ala
 678

15 (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 676 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Arg Val Leu Gly Gly Arg Cys Gly Ala Leu Leu Ala Cys Leu
 1 5 10 15

Leu Leu Val Leu Pro Val Ser Glu Ala Asn Phe Leu Ser Lys Gln
 20 25 30

25 Gln Ala Ser Gln Val Leu Val Arg Lys Arg Arg Ala Asn Ser Leu
 35 40 45

Leu Glu Glu Thr Lys Gln Gly Asn Leu Glu Arg Glu Cys Ile Glu
 50 55 60

30 Glu Leu Cys Asn Lys Glu Glu Ala Arg Glu Val Phe Glu Asn Asp
 65 70 75

Pro Glu Thr Asp Tyr Phe Tyr Pro Lys Tyr Leu Val Cys Leu Arg
 80 85 90

Ser Phe Gln Thr Gly Leu Phe Thr Ala Ala Arg Gln Ser Thr Asn
 95 100 105

35 Ala Tyr Pro Asp Leu Arg Ser Cys Val Asn Ala Ile Pro Asp Gln
 110 115 120

	Cys Ser Pro Leu Pro Cys Asn Glu Asp Gly Tyr Met Ser Cys Lys	125	130	135
	Asp Gly Lys Ala Ser Phe Thr Cys Thr Cys Lys Pro Gly Trp Gln	140	145	150
5	Gly Glu Lys Cys Glu Phe Asp Ile Asn Glu Cys Lys Asp Pro Ser	155	160	165
	Asn Ile Asn Gly Gly Cys Ser Gln Ile Cys Asp Asn Thr Pro Gly	170	175	180
10	Ser Tyr His Cys Ser Cys Lys Asn Gly Phe Val Met Leu Ser Asn	185	190	195
	Lys Lys Asp Cys Lys Asp Val Asp Glu Cys Ser Leu Lys Pro Ser	200	205	210
	Ile Cys Gly Thr Ala Val Cys Lys Asn Ile Pro Gly Asp Phe Glu	215	220	225
15	Cys Glu Cys Pro Glu Gly Tyr Arg Tyr Asn Leu Lys Ser Lys Ser	230	235	240
	Cys Glu Asp Ile Asp Glu Cys Ser Glu Asn Met Cys Ala Gln Leu	245	250	255
20	Cys Val Asn Tyr Pro Gly Gly Tyr Thr Cys Tyr Cys Asp Gly Lys	260	265	270
	Lys Gly Phe Lys Leu Ala Gln Asp Gln Lys Ser Cys Glu Val Val	275	280	285
	Ser Val Cys Leu Pro Leu Asn Leu Asp Thr Lys Tyr Glu Leu Leu	290	295	300
25	Tyr Leu Ala Glu Gln Phe Ala Gly Val Val Leu Tyr Leu Lys Phe	305	310	315
	Arg Leu Pro Glu Ile Ser Arg Phe Ser Ala Glu Phe Asp Phe Arg	320	325	330
30	Thr Tyr Asp Ser Glu Gly Val Ile Leu Tyr Ala Glu Ser Ile Asp	335	340	345
	His Ser Ala Trp Leu Leu Ile Ala Leu Arg Gly Gly Lys Ile Glu	350	355	360
	Val Gln Leu Lys Asn Glu His Thr Ser Lys Ile Thr Thr Gly Gly	365	370	375
35	Asp Val Ile Asn Asn Gly Leu Trp Asn Met Val Ser Val Glu Glu	380	385	390

-62-

Ile Arg Ala His Ser Cys Pro Ser Val Trp Lys Lys Thr Lys Asn
665 670 675

Ser
676

5 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
10 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCAAGACAAT GGAACCCAGG 20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
15 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

20 CATGGAATTC GTGACCGAT GTGCGGCTGT GAGGAG 36

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 95 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
25 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCAAGGGCTA CTGCCACACT CGAGCTGCGC AGATGCTAGC CTCAAGATGG 50

CTGATCCAAA TCGATTCCGC GGCAAAGATC TTCCGGTCCT GTAGA 95

30 (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 103 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGCTTCTACA GGACCGGAAG ATCTTTGCCG CGGAATCGAT TTGGATCAGC 50

CATCTTGAGG CTAGCATCTG CGCAGCTCGA GTGTGGCAGT AGCCCTTGCT 100

5 GCA 103

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13 amino acids

(B) TYPE: Amino Acid

10 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Xaa Gln Val Leu Ile Arg Arg Xaa Arg Ala Asn Thr Leu

1 5 10 13

(2) INFORMATION FOR SEQ ID NO:9:

15 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids

(B) TYPE: Amino Acid

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

20 Ala Asn Thr Leu

1 4

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 base pairs

25 (B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GATATCGATC CATGGCCCTC TCGCTCTC 28

30 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 31 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CATGGATCCT ACCGGAAGTC AAACACAGCT A 31

5 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GATATCGATG AGTGTGAAGT CCTTGTC 28

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

20 GTCGGATCCG ACAGAGACTG AGAAGCC 27

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala Phe Gln Val Phe Glu Glu Ala
1 5 8

(2) INFORMATION FOR SEQ ID NO:15:

30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCTGCTCGA GCGCTGTTG CCGGCGC 27

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

10 AGCTGCTCGA GGCAAATTCT TTACTTGAA 29

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- 15 (A) LENGTH: 30 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCTGCTCGA GGACCAAGTGC ACGCCCAACC 30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 28 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CGTGCTCGAG GACATCTTGC CGTGC GTG 28

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- 30 (A) LENGTH: 21 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CATTCAITTA TGTCAAATTC A 21

(2) INFORMATION FOR SEQ ID NO:20:

- 5 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGGAGATCA AGGTCTG 17

10 (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
15 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CATCTTGAGG CTAGCGGCTG CGGCGGGCTC CAC 33

(2) INFORMATION FOR SEQ ID NO:22:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

25 GTCGGTGACC GCTGCTGCGG GCTCCAC 27

(2) INFORMATION FOR SEQ ID NO:23:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GAAATTACAG ATCCGCAGCC CCGGGATGGG GCCGGCCCCG CTGCCGCTGC 50

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCTTGGATTG TAGCAAGCAC GACTGAAGGA GCCCATCAG TAGCACCTTT 50

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TCTTAAATTT AAGCTTCAGC TGCTCCTTGA TATTAACCTT TGTACAGAGT 50

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GCGCTGGCA ACGCG 15

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GTGACCGCGT TGCCAG 16

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: Nucleic Acid
5 (C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGAATTCCTC GAGCCGGGAC CTTTCCAGG GAGC 34

(2) INFORMATION FOR SEQ ID NO:29:

- 10 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CCAACTGTGT GTTTGAAGGC AAGAGCGG 29

WHAT IS CLAIMED IS:

1. Variant gas6 polypeptide which lacks one or more glutamic acid residues from the A domain of native gas6.
2. The variant gas6 of claim 1 which lacks the A domain of native gas6.
- 5 3. The variant gas6 of claim 2 which is the D domain of gas6.
4. The variant gas6 of claim 2 which is a G domain of gas6.
5. The variant gas6 of claim 1 which is derived from human gas6.
6. A composition comprising the variant gas6 of claim 1 and a physiologically acceptable carrier.
7. Nucleic acid encoding the gas6 variant of claim 1.
- 10 8. A vector comprising the nucleic acid of claim 7.
9. A host cell comprising the nucleic acid of claim 7.
10. A method of making variant gas6 polypeptide comprising culturing the host cell of claim 9 so that the nucleic acid is expressed and recovering the gas6 variant from the cell culture.
11. A method of making variant gas6 comprising:
 - 15 (a) culturing a host cell comprising nucleic acid encoding gas6 under conditions such that the nucleic acid is expressed and the gas6 polypeptide thus produced is essentially not γ -carboxylated, and
 - (b) recovering the gas6 variant from the cell culture.
12. The method of claim 11 wherein the culturing is carried out in the absence of Vitamin K.
13. The method of claim 11 wherein the host cell is deficient in γ -carboxylase enzyme.
- 20 14. The method of claim 13 wherein the host cell is non-mammalian.
15. A method of activating Rse receptor which comprises the step of exposing a cell comprising the Rse receptor to exogenous gas6 polypeptide in an amount effective to activate the Rse receptor.
16. The method of claim 15 wherein the gas6 comprises human gas6.
17. The method of claim 15 wherein the gas6 comprises a variant gas6 which is essentially not γ -carboxylated.
- 25 18. The method of claim 15 wherein the gas6 comprises an immunoadhesin.
19. The method of claim 15 wherein the cell is a glial cell.
20. The method of claim 19 wherein the glial cell is a Schwann cell.
21. A method of enhancing survival, proliferation or differentiation of a cell comprising the Rse
- 30 receptor which comprises the step of exposing the cell to exogenous gas6 polypeptide in an amount effective to enhance survival, proliferation or differentiation of the cell.
22. The method of claim 21 wherein the gas6 is human gas6.
23. The method of claim 22 wherein the cell is a human cell.
24. The method of claim 21 which comprises enhancing proliferation of the cell.

25. An article of manufacture, comprising:
a container;
a label on said container; and
a composition contained within said container;
- 5 wherein the composition is effective for enhancing survival, differentiation or proliferation of a cell comprising the Rse receptor, the label on said container indicates that the composition can be used for enhancing survival, differentiation or proliferation of said cell, and the active agent in said composition comprises gas6 polypeptide.
26. A method of activating Mer receptor which comprises the step of exposing a cell comprising
the Mer receptor to exogenous gas6 polypeptide in an amount effective to activate the Mer receptor.
- 10 27. The method of claim 26 wherein the gas6 comprises human gas6.
28. The method of claim 26 wherein the cell is a mononuclear cell.
29. A method of enhancing survival, proliferation or differentiation of a cell comprising the Mer receptor which comprises the step of exposing the cell to exogenous gas6 polypeptide in an amount effective to enhance survival, proliferation or differentiation of the cell.

Gla Region Loop EGF-like Repeats SHBG-like Domain

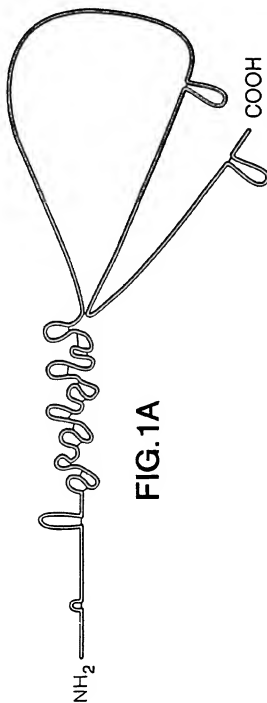


FIG. 1A

49	90	118	279	678		
A	B	C ₁	C ₂	C ₃	C ₄	D
						h Gas6

FIG. 1B

42	83	119	284	675		
68	21	45	56	46	59	38

b Protein S

FIG. 1C

42	83	119	284	676	
78	21	45	46	59	40

FIG. 1D

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C domain	mGas 6	D I D E C T I - D S D T C G D A R C K N L P G S Y S C L C D E G Y T I Y S S I K E K T I C Q
	hGas 6	D I D E C C A - D S E A C G E A R C K N L P G S Y S C L C D E G Y T I Y S S I K E K A C R
	hProts	D V D E C S L K P S I C G T A V I C K N T I P G D F E C I C P E G Y R V N L K S K S C E
	mGas 6	D V D E C C Q Q D R C E Q T C V N S P G S Y I C H C D G R G G L K L S P D M D T C E
	hGas 6	D V D E C C Q Q D R C E Q V C V N S P G S Y I C H C D G R G G L K L S P D M D T C E
	hProts	D V D E C C S E N M C A Q I C V N Y P G G V I C Y C D G R G G L K L S P D M D T C E
	mGas 6	D I L P C V P F S M A K S V K S L Y L G R M E S G T P V I
	hGas 6	D I L P C V P F S V A K S V K S L Y L G R M E S G T P V I
	hProts	V V S V L C L P L N L D K Y E L L Y L A E Q E A G V - V I
		→ G domain
		R L R E K R L Q P T R L L Y A E E D E R T E D P E G V L L F E
		V L K R E R L P E T S R F S A E E D E R T E D P E G V L L F E
	mGas 6	A G G R S D S T W I V L L G L R A G R L E L Q L R Y N G V G
	hGas 6	A G G R S D S T W I V L L A L R A G R L E L Q L R Y N G V G
	hProts	A E S I D H S A W L L L A L R G G K T I E V Q L K N E H S
		R I I T S S G P I T I N H G M W Q T I S V E E L E R N L V I K
		R V I T S S G P V I I N H G M W Q T I S V E E L E A R N L V I K
		K I I T T G G D V I N N G L W N M V S V E E L E H S I S I K
D domain	mGas 6	V N K D A V M K I A V A G E L F Q I E R G L - Y H L N L T
	hGas 6	V N R D A V M K I A V A G E L F Q I E R G L - Y H L N L T
	hProts	T A K E A V M D I N K P G P L E K P E N G L L L E T R V Y F
		V G G I I E K E S E L V Q P I N P R L D G C M R S W N M L
		V G G I P E H E S E L V Q P I N P R L D G C M R S W N M L
		A G G P P R K V E S E L T K P I N P R L D G C T R S W N L M

FIG.2B

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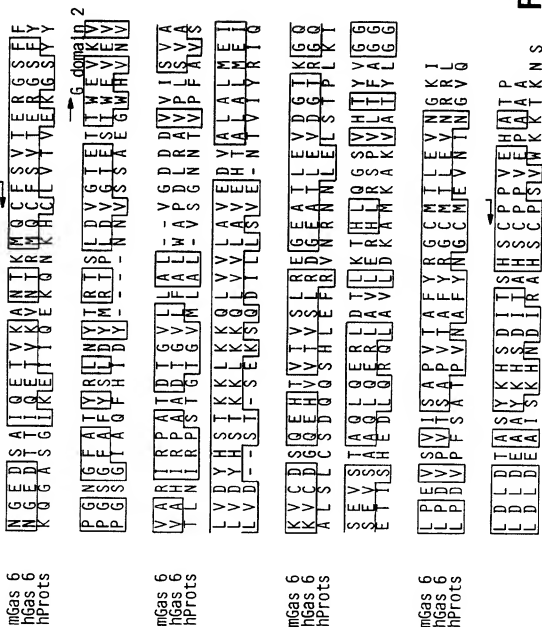


FIG.2C

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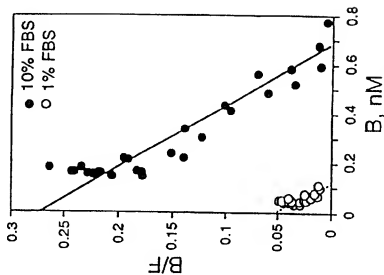


FIG.3C

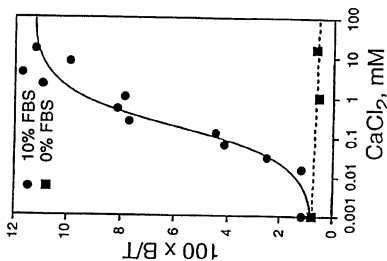


FIG.3B

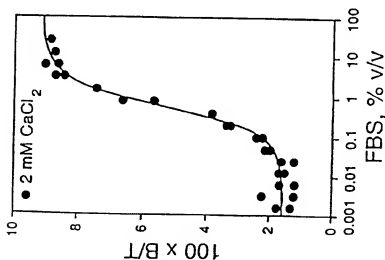


FIG.3A

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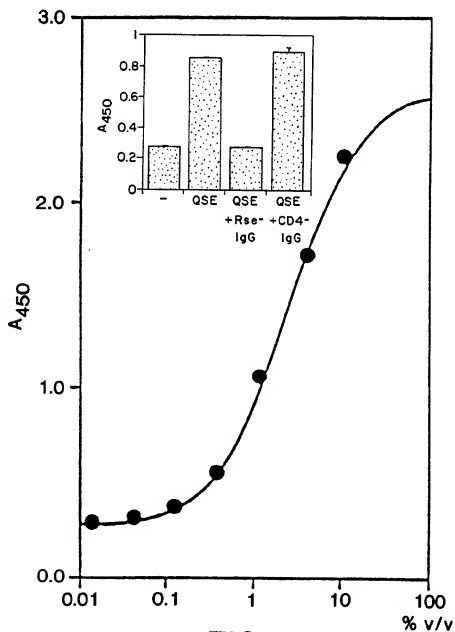


FIG.3D

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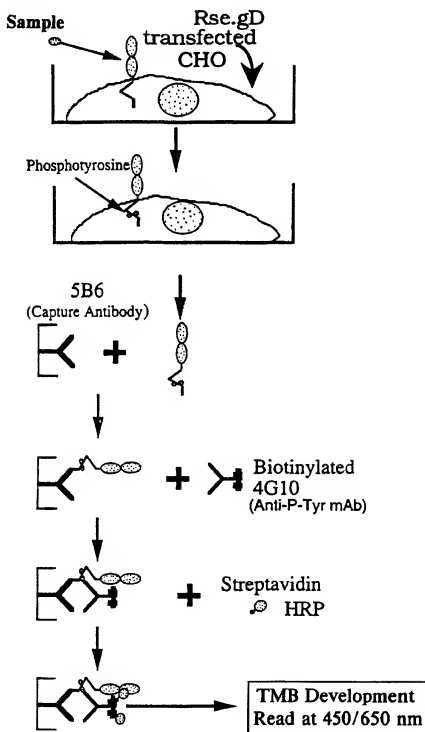


FIG.4

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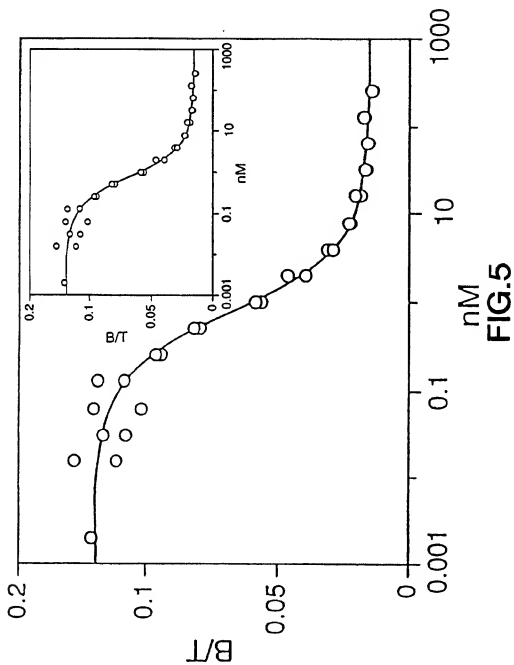


FIG. 5

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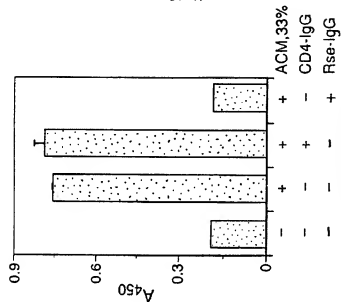


FIG. 6C

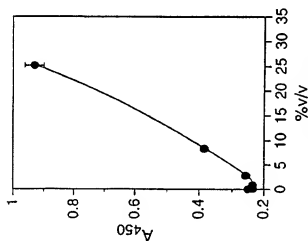


FIG. 6B

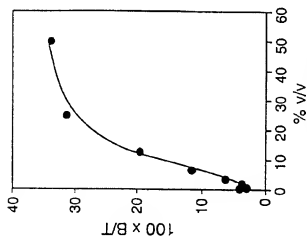


FIG. 6A

	Rse-ECD Binding					KIRA Activation	
	A	B	C	G1	G2		
gas6.gD						++	+
gD.gas6						++	+
gD.gas6.118-C						++	+
gD.gas6.279-C						++	+
gD.protein S						-	-

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FIG.7

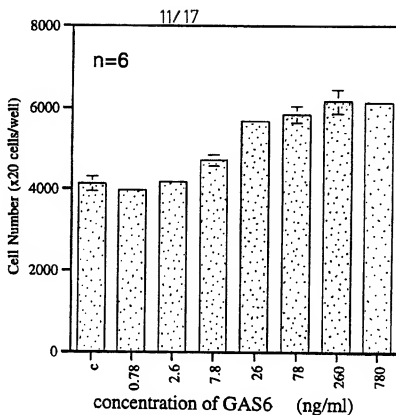


FIG.8

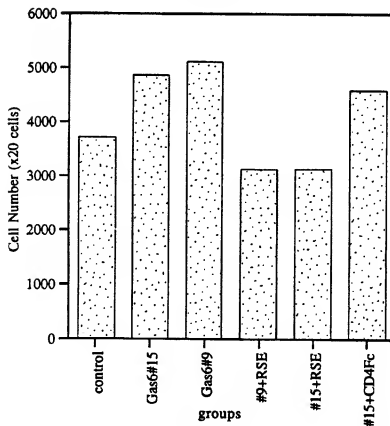


FIG.9

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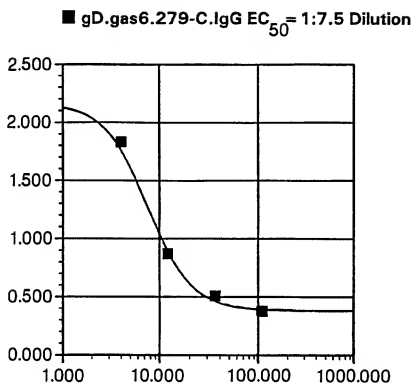


FIG. 10

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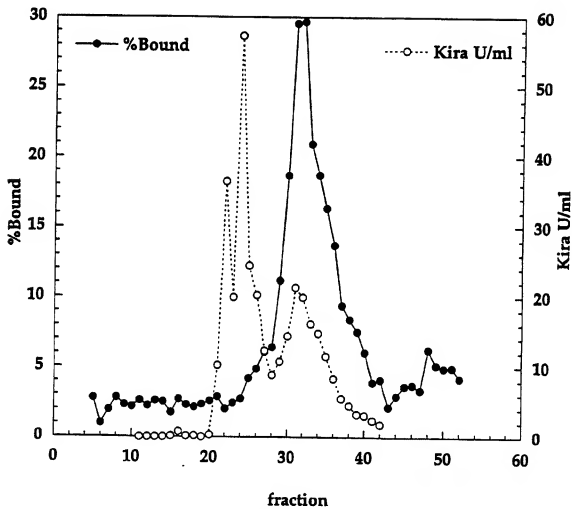
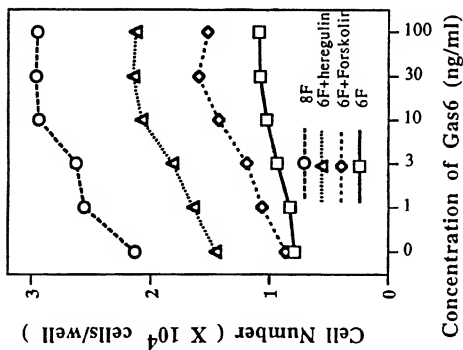
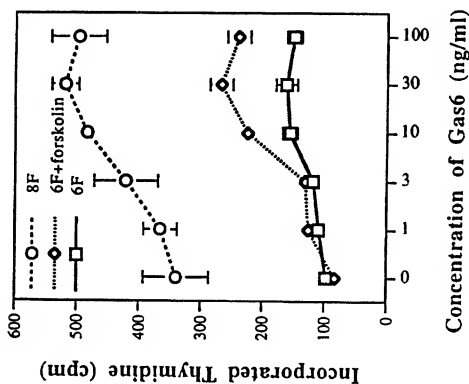
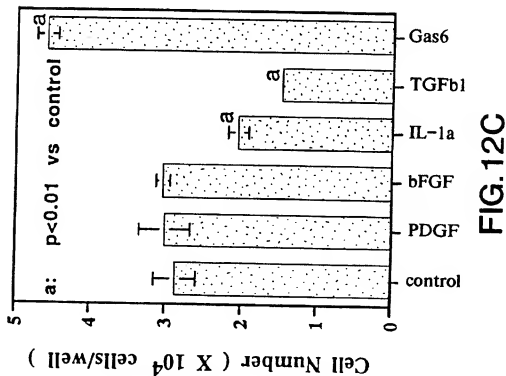
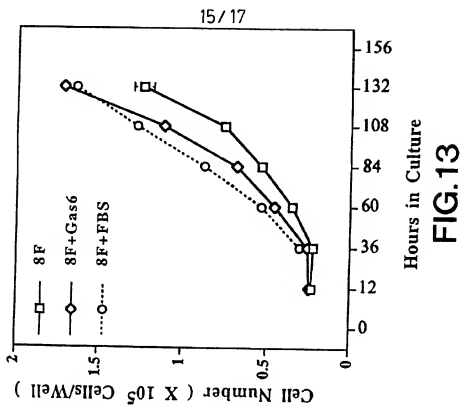


FIG. 11

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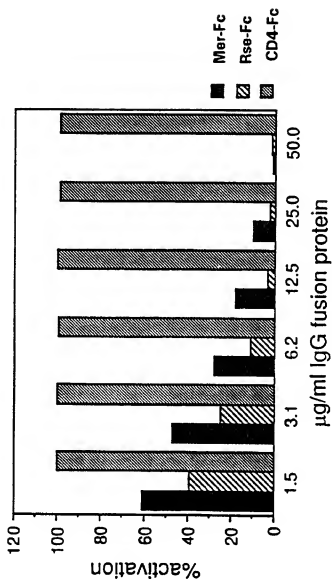
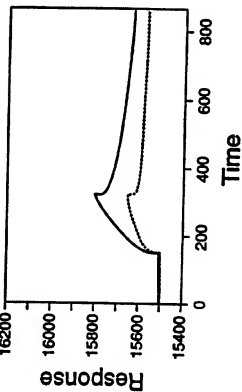
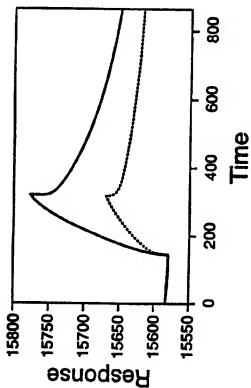
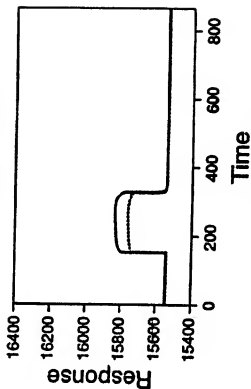
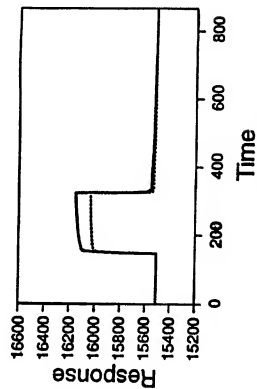


FIG. 14

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INTERNATIONAL SEARCH REPORT

International Application No.
PC/US 96/03031

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/12 C12N15/63 C12N15/85 C07K14/745 A61K38/36

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NATURE (LONDON) (1995), 373(6515), 623-6 CODEN: NATUAS; ISSN: 0028-0836, 16 February 1995, XP002008130 VARNUM, BRIAN C. ET AL: "Ax1 receptor tyrosine kinase stimulated by the vitamin K-dependent protein encoded by growth-arrest-specific gene 6" cited in the application see page 625, left-hand column, paragraph 3 - page 626, left-hand column, paragraph 1 --- -/-	1,15,16, 21-24, 26-29

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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- *Z* document member of the same patent family

Date of the actual completion of the international search

11 July 1996

Date of mailing of the international search report

25. 07. 96

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Authorized officer

Fuhr, C

INTERNATIONAL SEARCH REPORT

International Application No
PC./US 96/03031

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL (CAMBRIDGE, MASS.) (1995), 80(4), 661-70 CODEN: CELLS;ISSN: 0092-8674, 24 February 1995, XP002008131 STITT, TREVOR N. ET AL: "The anticoagulation factor protein S and its relative, Gas6, are ligands for the Tyro 3/Axl family of receptor tyrosine kinases" cited in the application see page 666, right-hand column, paragraph 3 - page 668, left-hand column, last paragraph ---	1,15,16, 21-24, 26-29
A	MOL. CELL. BIOL. (1993), 13(8), 4976-85 CODEN: MCEBD4;ISSN: 0270-7306, 1993, XP000574877 MANFIOLETTI, GUIDALBERTO ET AL: "The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade" cited in the application see page 4979, left-hand column, paragraph 3 - page 4981, right-hand column, paragraph 1; figure 3 see page 4982, right-hand column, paragraph 3 - page 4984, left-hand column, paragraph 1 ---	1,15,21
A	CELL, vol. 54, no. 6, 9 September 1988, NA US, pages 787-793, XP002008133 C. SCHNEIDER ET AL.: "Gene Specifically Expressed at Growth Arrest of Mammalian Cells" cited in the application see page 787, right-hand column, paragraph 4 - page 788, right-hand column, paragraph 1; table 2 ---	1,15
P,X	J. BIOL. CHEM. (1995), 270(39), 22681-4 CODEN: JBCHA3;ISSN: 0021-9258, 29 September 1995, XP002008134 OHASHI, KAZUMASA ET AL: "Stimulation of Sky receptor tyrosine kinase by the product of growth arrest-specific gene 6" see page 22682, left-hand column, paragraph 4 - page 22684, right-hand column, paragraph 1 ---	1,15,16, 21-24, 26-29

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INTERNATIONAL SEARCH REPORT

International Application No.

PC1/US 96/03031

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>ONCOGENE (1996), 12(3), 471-80 CODEN: ONCNES;ISSN: 0950-9232, 1 February 1996, XP000574912 GORUPPI, SANDRO ET AL: "Gas6, the ligand of Axl tyrosine kinase receptor, has mitogenic and survival activities for serum starved NIH3T3 fibroblasts" see page 472, left-hand column, paragraph 2 - right-hand column, paragraph 1 see page 477, left-hand column, paragraph 2 - page 478, left-hand column, paragraph 2 ---</p>	<p>1,15,16, 21-24, 26-29</p>
P,A	<p>J. BIOL. CHEM. (1995), 270(11), 5702-5 CODEN: JBCHA3;ISSN: 0021-9258, 17 March 1995, XP002008136 NAKANO, TORU ET AL: "Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for Ca2+-mobilizing growth factors" see page 5704, left-hand column, paragraph 2 - page 5705, right-hand column, paragraph 3; figure 4 ---</p>	<p>1</p>
P,A	<p>CELL (CAMBRIDGE, MASS.) (1995), 82(3), 355-8 CODEN: CELLB5;ISSN: 0092-8674, 11 August 1995, XP002008137 GODOWSKI, PAUL J. ET AL: "Reevaluation of the roles of protein S and Gas6 as ligands for the receptor tyrosine kinase Rse/Tyro 3" see the whole document -----</p>	<p>1,15,21, 26</p>

INTERNATIONAL SEARCH REPORT

ernational application No.

PCT/US 96/ 03031

Box I Observations where certain claims were found unsearchable (Continuation of item I of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15-24 and 26-29 are related to an in vivo method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. claims 1-14, 17 compl., 15, 16, 18-29
2. claims 15, 16, 18-29 partially

For further information, please see continuation sheet!

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Yes: Claims 1-14, 17 completely; 15, 16, 18-29 partially;

A variant gas polypeptide lacking γ -carboxylic acid residues compare to wild type; DNA coding for it; vectors and host cells comprising the DNA; methods of producing it; methods of activating receptors and influencing cells bearing said receptors; an article comprising it.

Yes: Claims 15, 16, 18-29 partially

methods of activating receptors and influencing cells bearing said receptors using gas6 proteins different from the variants lacking γ -carboxylic acid residues; an article comprising gas6 proteins.

Erythropoietin Structure-Function Relationships

MUTANT PROTEINS THAT TEST A MODEL OF TERTIARY STRUCTURE*

(Received for publication, February 12, 1993, and in revised form, April 2, 1993)

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On the basis of its primary sequence and the location of its disulfide bonds, we propose a structural model of the erythropoietic hormone erythropoietin (Epo) which predicts a four α -helical bundle motif, in common with other cytokines. In order to test this model, site-directed mutants were prepared by high level transient expression in Cos7 cells and analyzed by a radioimmuno assay and by bioassays utilizing mouse and human Epo-dependent cell lines. Deletions of 5 to 8 residues within predicted α -helices resulted in the failure of export of the mutant protein from the cell. In contrast, deletions at the NH₂ terminus (Δ 2-5), the COOH terminus (Δ 163-166), or in predicted interhelical loops (AB: Δ 32-36, Δ 53-57; BC: Δ 78-82; CD: Δ 111-119) resulted in the export of immunologically detectable Epo mutants that were biologically active. The mutant Δ 48-52 could be readily detected by radioimmunoassay but had markedly decreased biological activity. However, replacement of each of these deleted residues by serine resulted in Epo mutants with full biological activity. Replacement of Cys⁵⁹ and Cys⁵⁵ by tyrosine residues also resulted in the export of fully active Epo. Therefore, this small disulfide loop is not critical to Epo's stability or function. The properties of the mutants that we tested are consistent with our proposed model of tertiary structure.

Humoral regulation of red blood cell production was first proposed at the beginning of this century (1). Convincing physiologic experiments documenting the existence of erythropoietin (Epo)¹ (2-5) were followed by its purification (6) and partial structural characterization (7). The molecular cloning of this biologically and clinically important cytokine (8, 9) has led to further understanding of its properties (10, 11).

The binding of Epo to its cognate receptor (12) on erythroid progenitors in the bone marrow results in salvaging these cells from apoptosis (13), allowing them to proliferate and differentiate into circulating erythrocytes. The Epo receptor is a member of an ever enlarging family of cytokine receptors (14). In like manner, Epo shares weak sequence homology with

other members of a family of cytokines which also include growth hormone, prolactin, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, G-CSF, GM-CSF, M-CSF, oncostatin M, leukemia inhibitory factor, and ciliary neurotrophic factor (15-17). The genes encoding these proteins have similar numbers of exons as well as a clear relationship between intron-exon boundaries and predicted α -helical structure. These similarities have led to the prediction that this family of cytokines share a common pattern of folding into a compact globular structure consisting of four amphipathic α -helical bundles. Such theoretical models of the structures of human growth hormone (18) and IL-4 (19) have been in remarkably good agreement with subsequent structures established by x-ray diffraction (human growth hormone) (20, 21) or by multidimensional NMR (IL-4) (22, 23). Moreover, the crystal structures of GM-CSF (24) and monomeric M-CSF (25) are also in reasonable agreement with their predicted structures.

Thus far, the structure of Epo has not been analyzed by either x-ray diffraction or by NMR. In order to begin to gain an understanding of structure-function relationships, we have taken a three-pronged approach.

(a) Sequence determination of Epo from mammals of different orders in order to establish regions of homology (26).
(b) Construction of a model of the three-dimensional structure of Epo, followed by the design and preparation of mutants that test this model. These experiments are presented in this paper.

(c) Design and testing of mutants that provide information on receptor binding domain(s). This work will be presented in a subsequent paper.

MATERIALS AND METHODS

Computer-based Modeling of Structure

Prediction of Secondary Structure—Epo sequences from human, monkey, mouse, rat, sheep, pig, and cat were aligned (26) and examined using a hierarchical approach to secondary structure prediction that assumes that these proteins are members of the α/α folding class (27). First, the pattern-based method of Cohen *et al.* (28) for turn prediction was used to delimit sequence blocks likely to contain secondary structure. Predictions using the methods of Garnier *et al.* (29) and Chou and Fasman (30) suggested α -helical regions within these blocks. Finally, helical wheel projections were used to examine and then limit helix length based on preserving amphipathic character as codified in the work of Presnell *et al.* (31). The locations of glycosylation sites were also used to suggest helix boundaries.

Tertiary Structure Prediction—Earlier investigations have revealed the general principles of helix-to-helix packing in globular proteins (32). Exploring these principles, Cohen *et al.* (33) developed a method for the generation of three-dimensional protein structures from the secondary structure assignment. These methods have been applied to myoglobin, tobacco mosaic virus coat protein, growth

* This work was supported by National Institutes of Health Grants RO1-HL42949 (to H. F. B.) and RO1-GM 39900 (to F. E. C.) and by a grant from the R. W. Johnson Pharmaceutical Research Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The abbreviations used are: Epo, erythropoietin; IL, interleukin; bp, base pair(s); RIA, radioimmunoassay; IPTG, isopropyl-1-thio- β -D-galactopyranoside; WT, wild type.

‡ These algorithms are available from Dr. Cohen upon request.

hormone, α - and β -interferon, IL-2, and IL-4 (33-36).

The algorithm for tertiary structure generation is divided into four computations. The program *aspach* identifies clusters of hydrophobic residues within the putative helices that could mediate helix-helix interactions (32). *ApaFold* generates all possible helix pairings according to the location and geometric preferences of the interaction sites. *AbaFold* generates the three-dimensional models of all possible structures from the list of helix pairings (from *apaFold*) and subject to steric restrictions and geometric constraints on chain folding. In the final step, *avector* applies the user-defined distance constraints (e.g. disulfide bridges) to the structures generated. At this stage, coordinates have been specified only for residues in the core α -helices. For residues in sequentially distinct loops, lower bounds on the inter-residue distances can be inferred from the relevant helix terminus.

Preparation of Epo Mutants

Construction of the Mutagenic/Mammalian Expression Plasmid—A M13 plasmid, containing a 1.4-kilobase EcoRI-EcoRI human Epo cDNA insert (AHEPO PL12) was a gift from Genetics Institute (Cambridge, MA) (5). A 943 bp EcoRI-BglII fragment, corresponding to the complete coding sequence of the wild type human erythropoietin, including untranslated regions 216 bp upstream and 183 bp downstream, was inserted into the mammalian expression plasmid pSG5 (Stratagene) (37) and named pSG5-EPO/WT.

Site-directed Mutagenesis—was carried out according to the protocol described by Kunkel et al. (38). Single-stranded DNA was rescued from the pSG5-EPO/WT plasmid grown overnight in *Escherichia coli* CJ236, in 25 mM media containing 3M3K07 helper phage (In Vitrogen) and 70 μ M/ml kanamycin (Sigma). The resulting uracil-containing single-stranded DNA was used as a template for mutagenesis. Oligonucleotides (24–46-mer) were synthesized with their 5' and 3' ends complementary to the target wild type Epo sequence. A large variety of mutations (base substitutions, deletions and insertions) were created at the centers of the mutagenic primer sequences. Annealing of the phosphorylated primers (104 oligonucleotide/DNA template molar ratio) was performed in 10 μ l of a 20 mM Tris-HCl, pH 7.4, 2 mM MgCl₂, 50 mM NaCl solution. The reactions were incubated at 80 °C for 5 min and then allowed to cool slowly to room temperature over a 1-h period. The DNA polymerization was initiated by the addition as a mix of 1 μ l of 10 \times synthesis buffer (100 mM Tris-HCl, pH 7.4, 50 mM MgCl₂, 10 mM ATP, 5 mM each dNTPs, 20 mM dithiothreitol), 0.5 μ l of 8 units of T4 DNA ligase and 1 μ l (1 unit) of T4 DNA polymerase (Roehringer Mannheim). After 2 h at 37 °C, 80 μ l of 1 \times Tris-EDTA was added. 6 μ l of the diluted reaction mix was used to transform competent *E. coli* NM522 (ung⁻ Δ ur⁻).

Since a 40–80% mutation yield is normally obtained, four to five double-stranded plasmid clones from each reaction were sequenced with 7-deaza-dGTP and Sequences (U.S. Biochemical Inc.) (39). As a rule, the entire coding sequences of the Epo mutants were examined for the presence of unwanted mutation by sequencing or restriction enzyme mapping.

Production of Wild Type and Epo Mutants in Mammalian Cells—Cos7 cells grown to ~70% confluence were transfected with 10 μ g of recombinant plasmid DNA/10-cm dish using the calcium phosphate precipitation protocol (40). As a control of transfection efficiency, in several experiments 2 μ g of pCh110 plasmid (Pharmacia LKB Biotechnology Inc.) was cotransfected and β -galactosidase activity measured in the cytoplasmic extracts.

RNA Blot-hybridization Analysis—Total RNAs were prepared from transfected Cos7 cells (41) and 2- μ g samples electrophoresed on a 1.1% agarose gel containing 2.2 M formaldehyde. Transfer to GeneScreen Plus filters (Du Pont New England Nuclear) and hybridization with a ³²P-labeled WT Epo probe were carried out as previously described (42).

Quantitation of Transiently Expressed Recombinant Epos—The amount of secreted protein in the supernatants of transfected Cos7 was determined by a radioimmunoassay (RIA). The RIA was performed using a high titer rabbit polyclonal antiserum raised against the human wild type Epo and produced in our laboratory. [¹²⁵I]-labeled recombinant Epo was obtained from Amersham Corp. Details of the protocol have been published elsewhere (43).

Immunoprecipitation of ³⁵S-Labeled Epo Proteins—Three days after transfection, the Cos7 monolayers were washed extensively with 1 \times phosphate-buffered saline and the cells incubated for 20 min at 37 °C in 2 ml of Met³/Cys³ minimum essential media Eagle's modified medium. In each culture dish, 100 μ l of TRAN³⁵-LABEL ([³⁵S]cysteine and [³⁵S]methionine, ~100 nCi/ml, ICN Biochemical) was

then added. After 2 h, the conditioned media were harvested, and cellular extracts were prepared by lysis in radioimmune precipitation buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% (w/v) sodium azide, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1% (w/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1 μ g/ml aprotinin). Samples were preclashed with rabbit preimmune serum/protein A-Sepharose CL-4B (Pharmacia) for 2 h. Immunoprecipitations were performed overnight with our polyclonal antibody specific for human recombinant wild type Epo and immunoadsorbed with protein A-Sepharose CL-4B. Immunoprecipitates were run on 15% SDS-polyacrylamide gels (44) and analyzed by autoradiography after treatment with Enhance (Du Pont-New England Nuclear).

Bioassays—The dose-dependent proliferation activities of WT and Epo mutants were assayed *in vitro* using three different target cells: murine spleen cells, following a modification of the method of Krystal (45, 46); murine Epo-responsive MEL cell line, developed by Hankins (47); and human Epo-dependent UT-7/Epo cell line, derived from the bone marrow of a patient with acute megakaryoblastic leukemia (48). After 22–72 h of incubation with increasing amounts of recombinant proteins, cellular growth was determined by [³H]thymidine (Du Pont-New England Nuclear) uptake or by the colorimetric MTT assay (Sigma) (49).

Bacterial Expression—The wild type Epo target, corresponding to the nucleotide sequence coding for the mature protein, was polymerase chain reaction-amplified using appropriate primers. In the sense primer an *Nde*I site (CATATG) was placed immediately 5' to the Ala' codon of the mature protein. In the antisense primer a *Bgl*II site was placed 3' to the TGA stop codon. After enzymatic digestion, the 516-bp polymerase chain reaction fragment was inserted in an *Nde*I/*Bam*HI-cut pET16b plasmid (Novagen), which has a T7 promoter followed immediately by the lac operator. IPTG induction of transformed *E. coli* BL21(DE3)(T7 RNA polymerase⁺, lon⁻, ompT⁻) resulted in high levels of expression of a fusion protein with a 10-histidine stretch at the amino terminus. The oligo-His tag allowed the binding of the produced (His)₆-Epo to a nickel affinity resin and its elution by increasing imidazole concentrations in presence of phenylmethylsulfonyl fluoride (Sigma). Most of the produced protein formed insoluble aggregates and was solubilized and affinity-purified under denaturing conditions in 6 M guanidine HCl. Oxidative refolding was performed by overnight dialysis at 4 °C against 50 mM Tris-HCl, pH 8.0, 40 mM CuSO₄, and 2% (weight/volume) Sarkosyl. Soluble protein was dialyzed against 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM CaCl₂, and subjected to factor Xa (New England Biolabs) cleavage to remove the His₆-polyHis sequence. Monitoring of the fusion protein following induction and during the various steps of purification was done by electrophoresis on a 15% polyacrylamide-SDS gel, stained with Coomassie Brilliant Blue. Alternatively, the His₆-Epo fusion protein was detected on Western blot (50), using a 1/2000 dilution of our WT native Epo polyclonal antibody and a second biotinylated rabbit-specific antibody which is detected with a streptavidin-alkaline phosphatase conjugate (Amersham).

In Vitro Transcription/Translation—Sense and antisense primers, creating new *Bgl*II sites, respectively, 5' and 3' of the initiator and stop codons, were used in a polymerase chain reaction on pSG5-EPO/WT template. After *Bgl*II cleavage, the 594-bp polymerase chain reaction fragment was subcloned into pSP647 (51). This SP6-containing vector provides 5' and 3'-flanking regions from *Xenopus* β -globin mRNA, which allow efficient *in vitro* transcription/translation. Previous experiments showed poor yields of *in vitro* translated protein, when using the GC-rich natural 5' Epo untranslated region. One step *in vitro* transcription/translation was carried out by incubation of 1 μ g of circular p647-Epo in a 50- μ l reaction volume of SP6-TnT-coupled rabbit reticulocyte lysate system (Promega), in the presence of [³⁵S]cysteine (1200 Ci/mM, Du Pont-New England Nuclear). In some cases, canine pancreatic microsomal membranes were added to the reaction mix. A purified GST-human Epo receptor extracellular domain fusion protein (EREX) was a gift from W. Harris and J. Winkelman, and the binding of the ³⁵S-labeled translation products onto EREX-glutathione agarose beads was performed as described (52).

RESULTS

Construction of a Model of the Three-dimensional Structure of Erythropoietin

From an analysis of the putative Epo helix sequences, *aspach* identified eight possible helix-helix interaction sites.

TABLE I

Predicted α -helical regions of the mature erythropoietin protein

Data were obtained using the various algorithms for secondary and tertiary structure generations described under "Materials and Methods."

Helix	NH ₂ terminus	COOH terminus	Potential helix-helix interaction sites
A	9	22	19
B	59	76	63, 67, 70, 71
C	90	107	95, 102
D	132	152	141

In principle, these sites could be used to generate 1.6×10^4 structures. Of these, only 706 maintained the connectivity of the chain and were sterically sensible. These structures resembled four helix bundles, an increasingly common motif in protein structure (53). The structures that were not compatible with the native disulfide bridge between Cys₅ and Cys₁₆₁ were eliminated. This reduced the total number of structures from 706 to 184 (total computer time approximately 1 h on a Silicon Graphics IRIS 4D/35G). The remaining structures were then rank ordered by solvent-accessible surface contact area, a measure of the validity of model structures. The most compact structures were right handed, all anti-parallel four-helix bundles with no overhand connections, but this may be an artifact of a failure to add the polypeptide chain that forms the loops to the helical core constructed by *aabuild*. The other less compact structures were left-handed four-helix bundles with two overhand loops, a topology previously seen in the structures of IL-4 and growth hormone. We suspect that this is the likely structure for Epo. The consensus for assignments of putative α -helices in human Epo are summarized in Table I. First, analysis of the topological distribution of known four-helix bundle structures indicates that nearly all examples have an antiparallel orientation (53). Second, the left-handed four-helix bundles with two overhand connections arrange the four amphipathic helices to form a compact hydrophobic core. Finally, the AB and CD loop regions of Epo are predicted to have β -sheet segments analogous to IL-4 and growth hormone that preserves the compact globular nature of the Epo model structure. Fig. 1 shows schematic representations of predicted topological interactions between the four anti-parallel α -helical bundles.

Several authors have suggested that the helical cytokines form a structural superfamily (34, 54–57). On the basis of both the mature protein and the individual α -helices, Epo seems to be more closely related to growth hormone, prolactin, IL-6, and GM-CSF rather than the other members of the helical cytokine superfamily. Nevertheless, recent improvements in algorithms for the identification of distant evolutionary relationships between proteins from structural fingerprints suggested that it might be possible to align the IL-4 structure to the Epo sequences. The Eisenberg *et al.* (58) structural environment and 3D-1D profile methods are a powerful tool for recognizing that a sequence is compatible with a known structure, *e.g.* a four-helix bundle. The NMR structure of IL-4 from Smith *et al.* (59) was used to construct a 3D-1D profile. A mixture of sequences including four helix bundles, globins, and non-helical structures were aligned against the IL-4 profile. Not surprisingly, the IL-4 structures from human and mouse gave the highest scores ($Z^2 = 22.8$

and 8.1). However, the other known four-helix bundle cytokines known to share a similar fold with IL-4, *e.g.* human growth hormone (60) ($Z = 2.3$) and GM-CSF (61) ($Z = 2.3$) fared no better than some globin sequences (Kuroda's and slug sea hare globin, $Z = 5.0$ and 4.8) that adopt a distinct tertiary structure. The results for the human and sheep Epo sequences were also ambiguous ($Z = 1.6$ and 0.8). These results suggest that while profile methods are a powerful tool for recognizing structural similarity, their failure to identify homology does not exclude the possibility that two proteins share a common fold. For distantly related or unrelated structures, current profile methods cannot replace *de novo* methods for tertiary structure prediction.

Design and Expression of Epo Mutants That Test the Proposed Structure

To test the proposed four α -helical bundle structure of erythropoietin and at the same time to attempt to locate functional domains, we created by site-directed mutagenesis a series of deletion, insertion, and replacement mutants. These mutants were designed to analyze the principal predicted structural features of the molecule: α -helices, interconnecting loops, as well as the NH₂ and COOH termini. Structural and functional implications of the disulfide bridges and the glycosylation sites were also investigated.

α -Helices—Short amino acid deletions were prepared in, or close to, the predicted A, B, C, and D α -helices. Human wild type and mutants were transiently expressed in Cos7 cells. Northern blot analyses demonstrated that all the mutant plasmids produced about the same amount of mRNA as that of the wild type (data not shown). Yet, no detectable amount of Epo protein could be found in the Cos7 supernatants, either by radioimmunoassay or by bioassay using various Epo-dependent cell lines. Table II summarizes these findings.

An example of SDS-polyacrylamide gel electrophoresis of immunoprecipitates from *in vivo* ³⁵S labeling is presented in Fig. 2. As expected, when Cos7 cells were transfected with pSG5-EPO/WT, a 35–37 kDa band was detected in the supernatant. In contrast, the deletion mutants (Table II) could be detected in cellular extracts but were not exported from the cells. Fig. 2 shows the cytoplasmic retention of the mutant Δ 140–144, lacking 4 residues in the middle of the predicted D-helix. The apparent molecular mass (~ 28 kDa) is less than expected for a 5-amino acid deletion. Therefore, not only the secretion, but also the glycosylation, seem to be impaired. None of these mutants had deletion of glycosylation sites. It is likely that full glycosylation of Epo requires conservation of its molecular architecture. Similar results (reported in Table II) were obtained for all the mutants having partial deletion of an α -helical peptide segment.

Because contaminants in crude Cos7 cellular extracts severely interfere with the radioimmunoassay, no direct Epo quantitation was possible. However, aliquots of hypotonic extracts of Cos7 transfected with wild type Epo were able to sustain HDC57 proliferation. No similar biological activity was found for mutants with limited deletion of α -helices.

Interconnecting Loops—The peptide segment joining A- and B-helices presents several interesting features (Fig. 3A). AB loop consists of 36 amino acids. Two N-glycosylation sites and a small disulfide bridge are located in the first half and their biological implications will be discussed later. The COOH end of the AB loop contains a stretch of amino acids that is strongly conserved among mammals (26). Alignments of human, monkeys, cat, mouse, rat, pig, and sheep Epops showed a consensus sequence: DTKVNFYAWKR(M/I/E)/DJVG (residues 43–57). Three deletions were constructed:

³ Z scores are used to describe the normalized weight associated with a profile score. A distribution is built from a collection of sequences with a mean Z score of 0.0 and a standard deviation of 1.0. Z scores greater than 6.0 are associated with significant alignments. Z scores between 3.0 and 6.0 may or may not be structurally relevant.

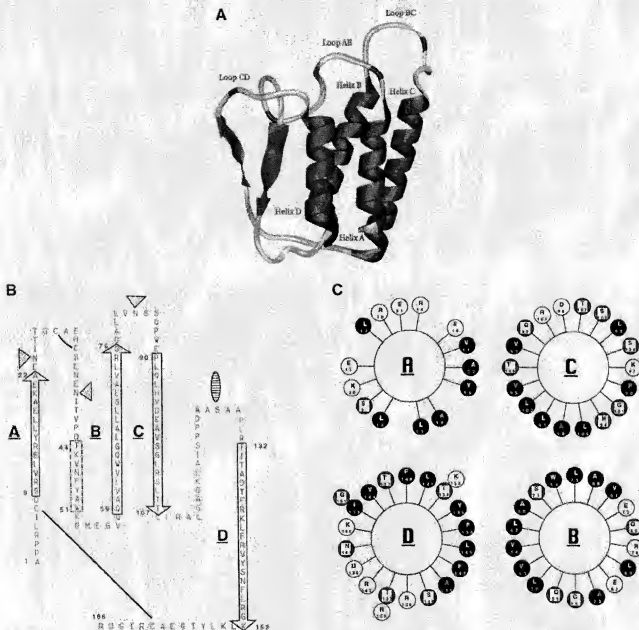


FIG. 1. Model of the three-dimensional structure of erythropoietin. A, ribbon diagram of the predicted Epo tertiary structure. The four α -helices are labeled A–D (*magenta*). Loops between helices are named for the helices they interconnect. Two regions of extended structure which could form hydrogen bonds between Loop AB and Loop CD are also presented (*cyan*). N- and O-glycosylation sites are indicated in green and blue, respectively. Disulfide bonds bridge residues 29–33 in Loop AB, and 7–161 on the NH₂-terminal side of Helix A and the COOH-terminal side of Helix D are not shown. N.B.: The loop tracing shown does not represent predicted coordinates. B, schematic representation of Epo's primary structure depicting predicted up-up-down-down orientation of the four antiparallel α -helices (boxes with arrowhead). This folding pattern is strongly suggested by the large size of the two interconnecting loops AB and CD. The limits of each helix were drawn according to Table I. A predicted short region of β -sheet is delineated by the dashed rectangle. The N-glycosylation sites are represented by the dotted diamonds, and the O-glycosylation site by the dashed oval. The locations of the two disulfide bridges are shown. C, cross-section of the Epo molecule at the level of the four α -helices. The helical wheel projections are viewed from the NH₂ end of each helix. The hydrophobic residues, localized inside the globular structure, are indicated by filled circles. The charged and neutral residues (open and gray circles, respectively) are exposed at the surface of the molecule.

$\Delta 43$ –47, $\Delta 48$ –52, and $\Delta 53$ –57, and transiently expressed in Cos7. The amount of mutants detected by RIA in the supernatants of transfected cells was 10–40% lower than observed with wild type Epo (Fig. 3B). Nevertheless, the three secreted mutants were biologically active. However, because $\Delta 48$ –52 exhibited a marked decrease of the specific bioactivity, this site was studied in more detail by means of serine replace-

ments. Krystal *ex vivo* bioassay as well as HCD57 and UT7-Epo *in vitro* bioassays showed that these Ser mutants had biological activities similar to that of wild type (Fig. 3C). Therefore, the observed decreases in both RIA and bioassay for the three deletion mutants are likely to be the result of changes of structural conformation. The long length of loop AB may be critical for the up-up-down-down topography. A

TABLE II

Short deletions in, or close to, α -helicesPredicted NH₂ and COOH termini of each α -helix are indicated in the vertical boxes.

Mutants			
$\Delta 12-16$	9	A helix	
	22		
$\Delta 65-69$	59	B helix	RNA levels comparable to WT.
	76		
$\Delta 96-100$	90	C helix	No detectable Epo in the Cos7 supernatant, both by RIA and bioassay.
$\Delta 105-109$	1		
	107		
$\Delta 122-126$			
$\Delta 131-135$	132	D helix	
$\Delta 140-144$	1		
$\Delta 142-150$			
$\Delta 152-155$	152		
$\Delta 156-160$			

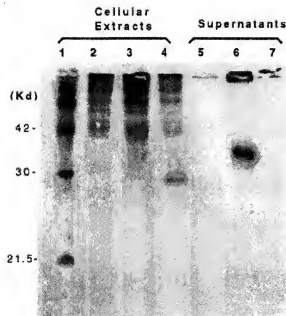


Fig. 2. Immunoprecipitations of wild type Epo and the $\Delta 140-144$ mutant. Cos7 cells were transfected with pSG5-EPO/wt, or pSG5-EPO/ $\Delta 140-144$. After 3 days, the cells were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine. Immunoprecipitations of cellular extracts and supernatants were performed with our polyclonal antibody, raised in rabbit against the native human Epo. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes 2-4 correspond to the cellular extracts; lanes 5-7 in the culture supernatants from transfected Cos7 with: plasmid without insert (lanes 2 and 5), wild type Epo (lanes 3 and 6), and $\Delta 140-144$ (lanes 4 and 7). Lane 1 represents the protein molecular weight standard. The two arrows show the normal secretion of the wild type Epo (35-37 kDa) and the cytoplasmic retention of the mutant $\Delta 140-144$ (~28 kDa).

shorter AB segment may impose a strain on the interhelical connection. Chou and Fasman (30) algorithms predicted a short β -sheet structure from residues 44 to 51 (<P_{ab}><P_b>, 1.005 < 1.196). The presence of a short region of β -sheet in the connection between helices 1 and 2 (A and B) have been documented in the analyses of the three-dimensional structures of IL-4 (22, 23), GM-CSF (24), and monomeric M-CSF (25). In contrast, in human GH a short segment of α -helix is

found at the same location (20). The structure/function implications of these short features are not yet understood.

Helix B is linked to helix C by a much shorter segment (residues 77-89) and contains in its center the third N-glycosylation site (Asn⁷⁸). When the $\Delta 78-82$ mutant was expressed, a secreted protein was detected in the conditioned medium and conferred proliferative bioactivity on Epo-dependent cell lines (see Fig. 8).

A similar long crossover connection (23 amino acids) is found between helix C and helix D. In contrast to what we previously observed for loop AB, a large deletion of 9 residues at position 111-119 or a 7-amino acid insertion of a myc epitope after residue 116 did not affect the secretion of these mutants (Fig. 4). Furthermore, these two proteins had normal specific activity, as seen by the ratio of bioassay to RIA. Our rabbit polyclonal antibody raised against the native form of the human wild type fully recognized the two mutants, demonstrating that the overall spatial conformation of Epo was well preserved. According to the algorithm of Emini *et al.* (62), the residues 111-119 are predicted to be at the surface of the molecule. Since the $\Delta 111-119$ mutant is readily secreted and has full biological activity, it seems unlikely that the putative β -sheet segment in the CD loop is an important determinant of molecular stability. Primary amino acid alignments of mammalian Epo showed a large variation in the sequence of residues 116-130, including amino acid deletion, insertion, and substitution (26). Surprisingly, when the deletion $\Delta 122-126$ mutant, which removed the O-glycosylation site (Ser¹²⁶), was transiently expressed in monkey cells, protein secretion was inhibited. Both rodents, rat and mouse, lack the O-glycosylation site because of a Ser¹²⁶ to Pro replacement. Furthermore, when a Ser¹²⁶ replacement mutant was expressed in normal Chinese hamster ovary cells, (63) or when wild type Epo was expressed in cells having a defect in O-linked glycosylation (64), neither secretion nor biological activity were impaired. Therefore, failure of secretion of the $\Delta 122-126$ mutant may be the result of some other structural alteration. In particular, the proline residue at position 122 is invariant among mammals.

NH₂ and COOH Termini—Deletion of residues 2 to 5 only slightly affected the processing of a biologically active protein (see Fig. 8). This deletion may impair cleavage of the propeptide, therefore explaining the lower yield of secreted Epo mutant in comparison to that of wild type. The fact that the mature monkey protein has an elongated (Val-Pro-Gly) NH₂ terminus strongly suggests that the NH₂-terminal part is not involved in the bioactivity of the molecule. Further evidence comes from the results, reported below, on the N-poly-His-Epo fusion protein expressed in *E. coli*, and also from the identical binding of *in vitro* translated ³⁵S-labeled wild type Epo onto EREX-glutathione agarose beads (Fig. 5), with or without addition of canine pancreatic microsomal membranes which permit cleavage of the propeptide.⁴

The COOH-terminal sequence following helix D can clearly be divided into two distinct domains, separated by Cys¹⁵¹. The residues 151-161 were of special interest because they are highly conserved among mammals (26). There are only two substitutions: Lys¹⁵⁴ is replaced by a Thr in artiodactyls and cat, and Ala¹⁶⁰ is replaced by a Val in mouse Epo. Both the $\Delta 152-155$ and the $\Delta 156-160$ mutants remained in the cytosol of the transfected Cos7 (Table II). One possible explanation is that the residues 152-160 may, in fact, participate in the D helix. We predict that Gly¹⁵³ is the break point of the struc-

⁴ All the mutants described in this paper were subcloned into pSG4T plasmid. Studies of the binding of their translation products to EREX are in process.

Model of Erythropoietin Structure

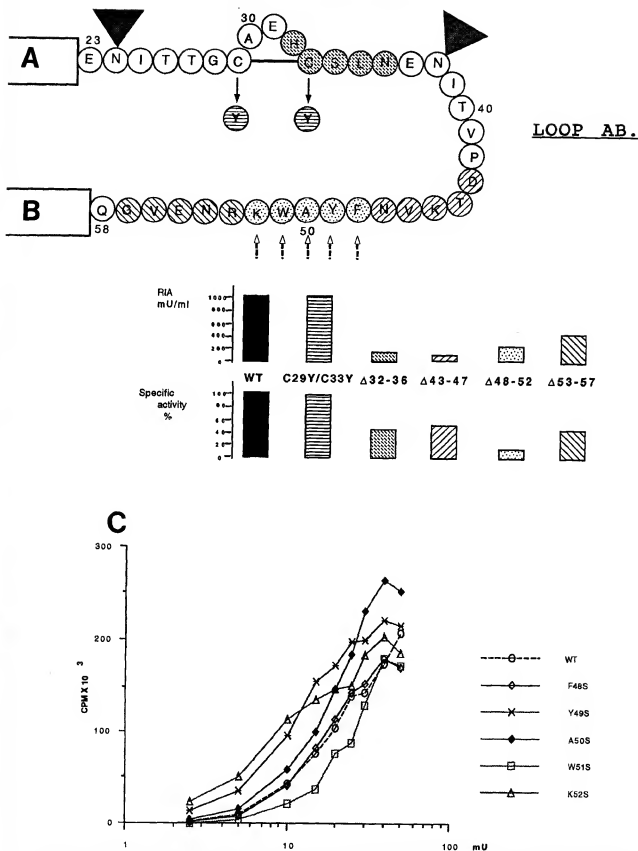


FIG. 3. Interconnecting loop AB. A, schematic representation of the loop AB showing the localization of mutants with various deletions and amino acid replacements. The dashed arrows point to the positions of the serine substitutions (in Δ48-52). The two N-glycosylation sites are represented by the gray diamonds. The small Cys³⁰=Cys⁴⁴ disulfide bridge is indicated. B, amount and biological activities of secreted

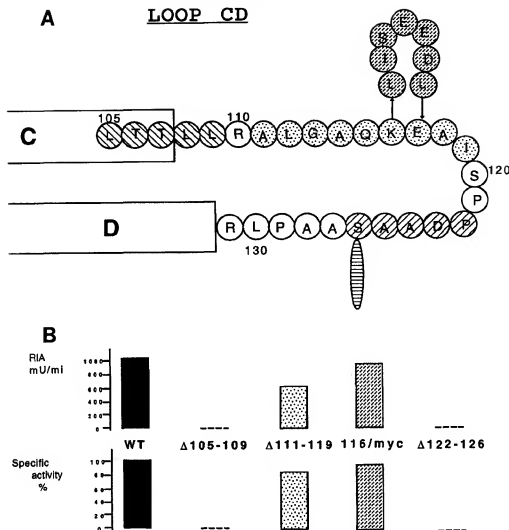


FIG. 4. Interconnecting loop CD. A, schematic representation of the loop CD showing the location of three deletion mutants: $\Delta 105-109$, $\Delta 111-119$, $\Delta 122-126$, and the insertion of 7 residues after Lys168 (myc epitope). The O-glycosylation site is indicated by the dashed oval. B, secretion and biological activities of the mutants located in loop CD. The two bar graphs were created as described in Fig. 3B. The two mutants $\Delta 111-119$ and 116/myc were normally secreted and had full biological activities. mU, millunit.

ture. However, it is possible that this residue causes only a bend in the α -helical structure and helix D may extend to Gly¹⁵⁸. The COOH-terminal part of the protein (residues 162-166) is clearly not involved in any structural or functional feature. Thus, the deletion of the 4 last amino acids or the replacement of residues 162-166 by either a KDEL sequence or a poly-histidine sequence⁶ did not modify the specific

⁶ The poly-His tail wild type mutant was purified by means of nickel affinity chromatography which enables quantitation of cytosolic-retained mutants. The (His)₆ COOH-terminal sequence has been appended to all the mutants described in this paper. Experiments are in progress to exploit this strategy.

activity of the erythropoietin (Fig. 6). Radioimmunoassay revealed that the secretion of the KDEL-tail mutant in the media of transfected cells was 45% less than normally obtained with the wild type Epo. However, when compared to the wild type, this mutant had more biological activity in the hypotonic Cos7 cell extracts. The KDEL COOH-terminal sequence has been shown to be essential for the retention of several proteins in the lumen of the endoplasmic reticulum (65). Nevertheless, because of overproduction in transiently expressed cells, a large percentage of recombinant protein escaped into the media.

Disulfide Bridges—Wang *et al.* (66) demonstrated that the biological activity of Epo was lost irreversibly if the sulphydryl

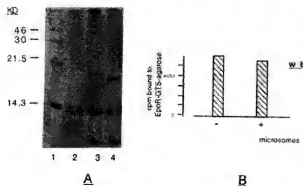


Fig. 5. *In vitro* translation of the Epo wild type. *A*, analysis of the ^{35}S -labeled translation products by SDS-polyacrylamide gel electrophoresis. One-step transcription/translation reactions were performed in the SP6-TnT rabbit reticulocyte lysate system. 1/30 of each reaction was resolved on a 15% polyacrylamide gel. Lane 1, low M_r standard from Amersham Corp.; lane 2, *in vitro* reaction without added plasmid; lanes 3 and 4, translation products obtained after incubation of $1 \mu\text{g}$ of circular p64T-Epo, respectively, in the presence or absence of canine pancreatic microsomal membranes. *B*, binding of the *in vitro* translated Epo wild type onto Epo receptor-GTAS-agarose beads. 6×10^6 count/min of purified ^{35}S -labeled erythropoietin products, processed with microsomes (+) or not (-) were incubated in the presence of EREX, following the protocol described by Harris *et al.* (65). Identical binding demonstrated that the conservation of the septide did not impair the hormone-receptor interaction.

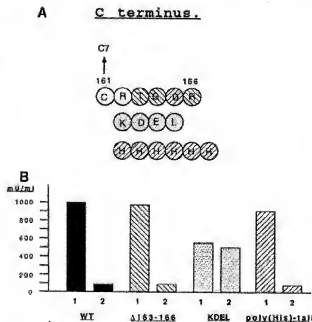


Fig. 6. COOH end of Epo. *A*, schematic representation of the analyzed mutants, corresponding to the deletion of the four last amino acids $\Delta 163$ -166 and the replacements of the residues 162-166 by a KDEL or poly(His) sequences. *B*, relative secretion of these mutants. The bioactivities in the supernatants (1) and the cell extracts (2) of transformed Cos7 cells were measured by *in vitro* proliferation assay using HCD57. More KDEL mutant remained in the cytosol of the Cos7, when compared with the wild type Epo and $\Delta 163$ -166 or poly(His) mutants. However, all the analyzed mutants had the same specific activity as that of the wild type. mIU, millunit.

groups were alkylated. The mature human Epo has two internal disulfide bonds: Cys²-Cys¹⁶³, linking the NH₂ and COOH termini of the protein and a small bridge between Cys²⁰ and Cys³³. Cys³³ was previously changed to Pro by site-directed

mutagenesis, and the resulting protein was reported to have greatly reduced *in vitro* biological activity (67). However, rat and mouse Epos have the same substitution and yet exhibit full cross-species bioactivity. To resolve the role of the small disulfide bridge in human Epo function, we created a C29Y/C33Y double mutation. The resulting mutant was normally processed and showed the same *in vitro* bioactivity as the wild type (Fig. 3B).⁶ Furthermore, the deletion of 5 amino acid residues ($\Delta 32$ -36) did not impair the secretion of a biologically active mutant. These data suggest that only the native and fully conserved disulfide bridge Cys²-Cys¹⁶³ is crucial for the preservation of the molecular structure of erythropoietin.

Functional Role of the Glycosylation—Natural or recombinant human Epo is a heavily glycosylated protein; 40% of its molecular weight is sugars (11). The protein has three *N*-linked oligosaccharide chains, located at amino acid positions 24 and 38 (in predicted loop AB) and position 83 (in loop BC). It has one *O*-linked carbohydrate chain at position 126 (in loop CD), which is missing in rodents. The role of these sugar chains in the biological activity of the human hormone has been extensively studied. Site-directed mutagenesis at the *N*-glycosylation sites demonstrated that even though the sugars were important for proper biosynthesis and secretion, their removal did not affect *in vitro* activity. This finding was corroborated by several investigators (68, 69). However, Takeuchi *et al.* (70) found that *N*-glycanase digestion results in almost complete loss of biological activity. In contrast, there is general consensus that glycosylation plays a key role in the biological activity of the hormone *in vivo*. Various reports have demonstrated that the *N*-linked sugar chains enhance the stability and survival of Epo in the blood stream (71, 72) and protect the hormone against clearance by the liver (73), thereby enabling the transit of the hormone from its site of production in the kidney to its target cells in the bone marrow (74).

We expressed the wild type Epo in *E. coli*. Accordingly, the produced protein completely lacks sugar. The pET expression system was used and is detailed under "Materials and Methods." IPTG induction of transformed BL21(DE3) bacterial strain rapidly results in a high level of expression of the poly-His Epo fusion protein (Fig. 7, *A* and *B*). After 3 h of induction, we obtained a typical yield of ~1 mg of total protein/ml of culture. However, the vast majority of the expressed protein was present in the inclusion bodies, and therefore its solubilization and purification on the nickel beads were performed in 6 M guanidine HCl. Oxidative refolding and factor Xa cleavage resulted in soluble forms (Fig. 7C), and the *in vitro* biological activity was tested on HCD57 cells. The cleaved *E. coli* recombinant Epo showed a notable decrease of the specific activity (10% less than the fully glycosylated mammalian expressed protein), but was still able to maintain HCD57 proliferation. The observed reduction of *in vitro* activity is likely to be due to improper refolding of the insoluble protein and impaired physical stability of the *E. coli* Epo as previously reported (55). However, the fact that the *E. coli* Epo was still able to trigger HCD57 growth indicated an overall preservation of the molecular structure. The uncleaved fusion protein, with 10 His residues at the NH₂ terminus, exhibited a 67% loss in biological activity when compared to the cleaved protein. Thus, the addition of a 20-residue sequence to the NH₂ terminus partially inhibited the biological activity.

⁶ Two single replacement mutants (C29Y and C33Y) were also stable and had full biological activity (results not shown).

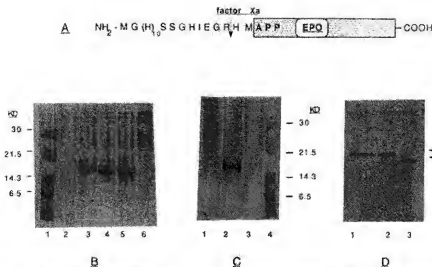


FIG. 7. Bacterial expression of wild type Epo. A, diagram of the fusion protein. An NH_2 -terminal 22-amino-acid long peptide, containing a 10 histidine stretch, was fused to the mature erythropoietin sequence. Factor Xa cleavage allowed the recovery of the mature Epo with only 2 extra residues at its amino terminus. B, IPTG induction of the fusion protein. Transformed *E. coli* BL21(DE3) cultures ($\text{OD}_{600} = 0.6$) were grown in the presence of 1 mM IPTG. Aliquots were collected at 0 (lane 2), 1 (lane 3), 2 (lane 4), and 3 h (lane 5) and analyzed on a 15% SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue. A high level of production of the fusion protein was rapidly obtained. Lane 6 corresponds to an aliquot from transformed bacteria grown for 3 h in a medium without IPTG. Lane 1 is a low molecular weight standard. C, purification of the fusion protein. After 3 h of IPTG induction, the produced $(\text{His})_{10}$ -Epo was solubilized in 6 M guanidine HCl and purified on a nickel affinity resin by increasing imidazole concentrations following the PET-His system protocol (Novagen). Samples of the column eluants were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. Lane 1, elution by 20 mM imidazole; lane 2, elution by 100 mM imidazole, releasing the fusion protein; lane 3, elution of the nickel by a 100 mM EDTA wash; lane 4, molecular weight standard. D, detection of the *E. coli* recombinant Epo on a Western blot. Solubilized proteins were separated on a 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with a 1/2000 dilution of our native wild type polyclonal antibody, as described under "Materials and Methods." Lane 1, analysis after oxidative reduction; lane 2, after dialysis against the factor Xa buffer; and lane 3, after factor Xa cleavage.

DISCUSSION

Currently, the accrual rate of new protein sequences through gene cloning far outstrips the rate of determination of three-dimensional structure. Epo is among a large number of biologically important proteins which have not yet been analyzed by x-ray diffraction or NMR. The problem is simplified by cumulative evidence that the structures of most proteins are likely to be variations on existing themes (27). Indeed, as mentioned above, Epo appears to share common structural features with a large group of cytokines (15–17).

Computer-based prediction of structure can be reduced to a three-stage process: secondary structure is predicted from the primary amino acid sequence and, when available, optical measurements. Analysis of Epo by circular dichroism reveals about 50% α -helix and no detectable β -sheet (7, 11). With the knowledge of disulfide bonds, secondary structural elements are then packed into a set of alternative tertiary structures. The number of plausible arrangements can be reduced by empirical knowledge of preferred helix-helix packing geometries and the need for globular structure to form a hydrophobic core. The putative tertiary structure is then refined by standard force field calculations. Since there are a large number of alternate tertiary structures, the availability of experimentally determined structure of a homologous protein is critically important. Thus, the predicted model of Epo structure gains considerable validity by knowledge of the structures of growth hormone (20, 21) and IL-4 (22, 23).

We have tested the predicted four anti-parallel α -helical bundle structure by means of site-directed mutagenesis. Deletions within predicted α -helices would be expected to destabilize tertiary structure, whereas deletions or insertions in non-helical segments should be permitted unless they impose

undue strain on the structure. For example, a deletion in an overhand inter-helical loop may result in insufficient length to connect the two helices. Results that we have obtained on mutants produced in mammalian (Cos7) cells are summarized in Fig. 8. Our measurements of the quantities of processed protein by RIA may underestimate the true amount of secreted Epo. Even a small deletion or insertion can result in a conformational change that may lead to impaired binding by our polyclonal antibody, raised against native human Epo. Thus the values for specific activity (biologic activity/RIA) that we report must be regarded as approximations. This caveat notwithstanding, our mutagenesis results are in good agreement with our proposed four α -helical model of erythropoietin. The proper folding of Epo into its native tertiary structure is necessary for stability and biological function. Mutations with short deletions inside predicted α -helices were not processed and exhibited no biological activity. In contrast, when deletions were created in predicted interconnecting loops, secreted proteins were detected, to varying degrees, both by radioimmunoassay and bioassay. Furthermore, additions or deletions at the NH_2 or COOH termini did not markedly impair the secretion and the biological activity of the Epo protein. Moreover, mutations at Cys³⁹ and Cys⁴⁰ showed that the small disulfide loop is not critical for biological activity. In order to delineate Epo's functionally important residues involved in the direct binding onto the Epo receptor, we have prepared and tested a series of amino acid replacements on the surfaces of the predicted α -helices. These experiments will be described in a subsequent paper.

Acknowledgments.—We thank Elizabeth Eltridge for technical assistance and Haana Bandos for secretarial and editorial assistance. We also thank Dr. David Hankins for providing the HC157 cell line.

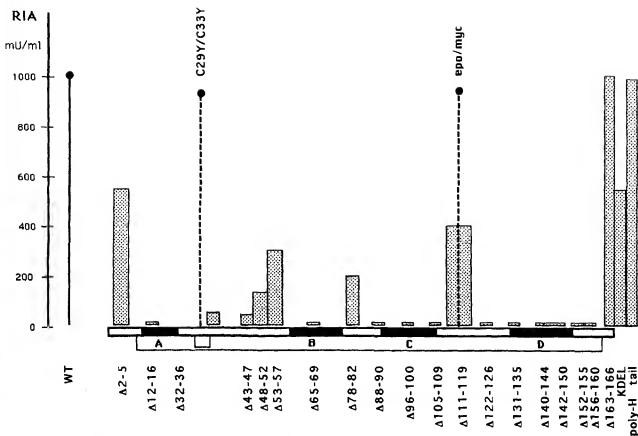


FIG. 8. Relationship between production of mutants and proposed secondary structure. This bar graph shows the amount of secreted proteins in the supernatants of transiently expressed Epo mutants, as detected by radioimmunoassay. The mutants were aligned over a schematic representation of the native Epo molecule. Each deletion is shown as a stippled bar, the width of which is proportional to the number of residues deleted. The four α -helices are represented by the black rectangles. The two disulfide bridges are indicated. These mutagenesis results are in good agreement with our proposed four α -helical model of Epo.

Dr. N. Komatsu for the UT-7/Epo cell line, and Dr. John Winkelman for the Epo receptor-agarose beads.

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US 2003/0077753A1

(19) **United States**(12) **Patent Application Publication**
Tischer(10) **Pub. No.: US 2003/0077753 A1**(43) **Pub. Date: Apr. 24, 2003**(54) **DIGLYCOSYLATED ERYTHROPOIETIN****Publication Classification**(76) **Inventor: Wilhelm Tischer, Peissenberg (DE)**(51) **Int. Cl.⁷** **C07K 14/575; C12P 21/04;**
C12N 5/06; A61K 38/24
(52) **U.S. Cl.** **435/69.6; 435/320.1; 435/325;**
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NUTLEY, NJ 07110(57) **ABSTRACT**(21) **Appl. No.: 10/241,356**(22) **Filed: Sep. 11, 2002**(30) **Foreign Application Priority Data****Sep. 25, 2001 (EP) 01122555.4**

This invention is directed to an erythropoietin mutein having in vivo biological activity for causing bone marrow cells to increase production of reticulocytes and red blood cells, in that the mutein is N-glycosylated at Asn38 and Asn83 but not N-glycosylated at Asn24. Such muteins have improved pharmaceutical properties.

DIGLYCOSYLATED ERYTHROPOIETIN

FIELD OF THE INVENTION

[0001] The invention relates to new variants of erythropoietin that are diglycosylated, methods for the production and use, as well as pharmaceutical compositions thereof.

BACKGROUND OF THE INVENTION

[0002] Erythropoiesis is the production of red blood cells which occurs to offset cell destruction. Erythropoiesis is a controlled physiological mechanism that enables sufficient red blood cells to be available for proper tissue oxygenation. Naturally occurring human erythropoietin (hEPO) is a glycoprotein containing 165 amino acids that is produced in the kidney and is the humoral plasma factor which stimulates red blood cell production. Human EPO stimulates the division and differentiation of committed erythroid progenitors in the bone marrow. Human EPO exerts its biological activity by binding to receptors on erythroid precursors. Naturally occurring human erythropoietin is an acidic glycoprotein present in low concentrations in plasma to stimulate replacement of red blood cells which are lost through aging.

[0003] Erythropoietin has been manufactured biosynthetically using recombinant DNA technology (Egrie, J. C., et al., *Immunobiol.* 72 (1986) 213-224) and is the product of a cloned human EPO gene inserted into and expressed in the ovarian tissue cells of the Chinese hamster (CHO cells). Naturally occurring human EPO is first translated to a 166 aa containing polypeptide chain with arginine 166. In a posttranslational modification, arginine 166 is cleaved by a carboxypeptidase. The primary structure of human EPO (165 aa and 166 aa) is shown in SEQ ID NO: 1 and SEQ ID NO: 2. There are two disulfide bridges between Cys7-Cys161 and Cys29-Cys33. The molecular weight of the polypeptide chain of human EPO without the sugar moieties is 18,236 Da. In the intact EPO molecule, approximately 40% of the molecular weight is accounted for by the carbohydrate groups (Sasaki, H., et al., *J. Biol. Chem.* 262 (1987) 12059-12076).

[0004] Because erythropoietin is essential in red blood cell formation, it is useful in the treatment of blood disorders characterized by low or defective red blood cell production. Clinically, EPO is used in the treatment of various ailments, for example, anemia in chronic renal failure patients (CRF) and in AIDS and cancer patients undergoing chemotherapy (Danna, R. P., et al., In: M. B. Garnick, ed. *Erythropoietin in Clinical Applications—An International Perspective*. New York, N.Y.: Marcel Dekker; 1990, pp. 301-324). However, the bioavailability of currently available protein therapeutics such as erythropoietin is limited by their short plasma half-life and susceptibility to protease degradation. These shortcomings prevent them from attaining maximum clinical potency.

[0005] Modifications of the amino acid sequence of EPO have been disclosed, for example, in a number of references including U.S. Pat. No. 4,835,260; WO 94/25055; WO 94/24160; WO 94/02611; WO 95/05465.

[0006] Both human urinary derived erythropoietin and recombinant erythropoietin (expressed in mammalian cells) contain three N-linked and one O-linked oligosaccharide

chains which together comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38 and 83 while O-linked glycosylation occurs at a serine residue located at position 126 (Lai, et al., *J. Biol. Chem.* 261 (1986) 3116; Broudy, V. C., et al., *Arch. Biochem. Biophys.* 265 (1988) 329). The oligosaccharide chains have been shown to be modified with terminal sialic acid residues. Enzymatic treatment of glycosylated erythropoietin to remove all sialic acid residues results in a loss of in vivo activity but does not affect in vitro activity (Lowy et al., *Nature* 185 (1960) 102; Goldwasser, E., et al., *J. Biol. Chem.* 249 (1974) 4202-4206). This behavior has been explained by rapid clearance of asialoerythropoietin from circulation upon interaction with the hepatic asialoerythropoietin binding protein (Morrell et al., *J. Biol. Chem.* 243 (1968) 155; Briggs, D. W., et al., *Am. J. Physiol.* 227 (1974) 1385-1388; Ashwell, G., and Kawasaki, T., *Methods Enzymol.* 50 (1978) 287-288). Thus, erythropoietin possesses in vivo biological activity only when it is sialylated to avoid its binding by the hepatic binding protein.

[0007] The role of the other components in the oligosaccharide chains of erythropoietin is not well defined. It has been shown that partially diglycosylated erythropoietin has greatly reduced in vivo activity compared to the glycosylated form but does retain in vitro activity (Dordal, M. S., et al., *Endocrinology* 116 (1985) 2293-2299). In another study, however, the removal of N-linked or O-linked oligosaccharide chains singly or together by mutagenesis of asparagine or serine residues that are glycosylation sites sharply reduces in vitro activity of the altered erythropoietin that is produced in mammalian cells (Dube, S., et al., *J. Biol. Chem.* 263 (1988) 17516-17521).

[0008] Oligonucleotide-directed mutagenesis has been used to prepare structural mutants of EPO lacking specific sites for glycosylation (Yamaguchi, K., et al., *J. Biol. Chem.* 266 (1991) 20434-20439; and Higuchi, M., et al., *J. Biol. Chem.* 267 (1992) 7703-7709). Cloning and expression of non-glycosylated EPO in *E. coli* is described by Lee-Huang, S., *Proc. Natl. Acad. Sci. USA* 61 (1984) 2708-2712; and in U.S. Pat. No. 5,641,663.

[0009] EPO 640 619 relates to analogs of human erythropoietin comprising an amino acid sequence which includes at least one additional site for glycosylation. The added sites for glycosylation may result in a greater number of carbohydrate chains, and higher sialic acid content, than human erythropoietin. Erythropoietin analogs comprising amino acid sequences which include the rearrangement of at least one site for glycosylation are also provided. Analogs comprising an addition of one or more amino acids to the carboxy terminal end of erythropoietin wherein the addition provides at least one glycosylation site are also included.

[0010] PEGylation of glycosylated EPO is described in WO 01/02017. Such molecules show an improved biological activity. WO 00/32772 and Francis, G. E., et al., *Int. J. Hem.* 68 (1988) 1-18, describe polyethylene glycol-modified non-glycosylated EPO. The molecules of WO 00/32772 are additionally modified at positions 166. Such molecules are described as not causing a significant increase in hemato-crit. The PEG-polymer portion consists of 1-5 polymer chains. WO 00/32772 suggests to control the degree and site of PEGylation by lowering the pH and reducing the PEG:

amine ratio. Reactions run at pH 7 and 1.5:1 molar ratio of PEG-aldehyde: amine groups, preferentially react with the N-terminal α -amino group.

[0011] In spite of the numerous modifications that are known for EPO, there still exists a need for further EPO mutants with modified properties, especially with modified clearance and simple, reproducible methods for its production.

SUMMARY OF THE INVENTION

[0012] The invention provides a new class of EPO mutants. The EPO mutants according to the invention have the in vivo biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells.

[0013] The invention provides an erythropoietin mutant which has retained the potential N-glycosylation sites at Asn24, Asn38, Asn83, is N-glycosylated at Asn38 and Asn83 but is not N-glycosylated at Asn24 and is preferably linked at the N-terminal amino group and/or the ϵ -amino group of Lys20 to poly(ethylene glycol) group(s) (PEG), preferably to alkoxy poly(ethylene glycol) group(s), more preferably to lower methoxypoly(ethylene glycol) group(s).

[0014] The mutants of this invention have the same uses as EPO. In particular, the mutants of this invention are useful to treat patients by stimulating the division and differentiation of committed erythroid progenitors in the bone marrow. In the same way EPO is used to treat patients.

[0015] The invention also provides an aqueous composition comprising an erythropoietin mutant having N-glycosylation sites at Asn38 and Asn83 but not at Asn24, and a pharmaceutically acceptable buffer thereof.

[0016] In yet another embodiment, this invention provides a pharmaceutical composition comprising an erythropoietin mutant or an aqueous composition as referred to above.

[0017] The present invention also includes a method for making erythropoietin mutants according to the invention, which comprises the production of a glycosylated EPO fragment consisting of the amino acids 26-165 (EPO 26-165) and subsequent chemically linking or fusing of said fragment with a non-glycosylated but preferably PEGylated EPO fragment consisting of the amino acids 1-28 (EPO 1-28).

[0018] This invention is also directed to a method of treating a disease relating to anemia in chronic renal failure patients or to AIDS and the treatment of cancer patients undergoing chemotherapy comprising administering to a patient in need thereof a therapeutically effective amount of a mutant or composition as referred to above.

DETAILED DESCRIPTION OF THE INVENTION

[0019] The term "erythropoietin" (EPO) refers to a protein having the sequence SEQ ID NO: 1 or SEQ ID NO: 2, or a protein or polypeptide substantially homologous thereto, whose biological properties relate to the stimulation of red blood cell production and the stimulation of the division and differentiation of committed erythroid progenitors in the bone marrow.

[0020] The term "substantially homologous" means that a particular subject sequence, for example, a mutant sequence,

varies from a reference sequence by one to five substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and subject sequences. However, as mentioned above, the potential glycosylation sites Asn24, Asn38 and Asn83 are retained.

[0021] Human EPO has one o-glycosylation site (at Ser126 of SEQ ID NO:1) and three N-glycosylation sites (at Asn24, Asn38 and Asn83 of SEQ ID NO:1). The glycosyl residues at these sites are sialylated and are important for the in vivo half-life and subsequently for the efficacy of EPO. Elimination of glycosylation sites Asn24 results in an EPO mutant with improved in vivo efficacy. However, such EPO mutants can only be produced according to the state of the art by replacement of Asn24 by another amino acid which cannot be glycosylated. Therefore, these mutants differ from naturally occurring EPO by having an amino acid modified at a sensible position of the sequence. Such EPO mutants with modified amino acid sequence and modified glycosylation are described, for example, by Nimitz, M., et al., *Eur. J. Biochem.* 213 (1993) 39-56; Sasaki, H., et al., *Biochemistry* 27 (1988) 8618-8626; Delorme, C., et al., *Biochemistry* 31 (1992) 9871-9876; Fibi, M. R., et al., *Blood* 85 (1995) 1229-1236; and WO 99/11781 (see also EP 0 411 678; Takeuchi, M., et al., *Proc. Natl. Acad. Sci. USA* 86 (1989) 7819-7822; EP 0 427 189; EP 0 409 113; Dube, S., et al., *J. Biol. Chem.* 263 (1988) 17516-17521; Yamauchi, K., et al., *J. Biol. Chem.* 266 (1991) 20434-20439; EP 0 640 619; and Fibi, M. R., et al., *Applied Microbiol. Biotechnol* 35 (1991) 622-630).

[0022] Therefore, glycosylation to Asn24 can be prevented only by deleting, modifying or substituting this amino acid. Erythropoietin mutants which have retained the amino acids Asn24, Asn38 and Asn83, but are only glycosylated at Asn38 and Asn83, and not glycosylated at Asn24, are not known in the art and there is nothing in the art to provide guidance on how to produce such molecules.

[0023] The present invention provides a simple method for the production of EPO mutants which retain glycosylation, preferably natural glycosylation, in the C-terminal part of the molecule beginning with Cys29, being not glycosylated in the N-terminal part up to Gly28, whereby, in addition, said N-terminal part can be modified very easily and in a broad manner, if desired. Such a modification is, for example, a reproducible and defined attachment of side chains such as polyethylene glycols.

[0024] The term "N-terminal EPO fragment" or "EPO 1-28" refers to an EPO fragment having amino acids 1-28 of SEQ ID NO: 1 or SEQ ID NO: 2. As mentioned above, the term also comprises fragments with slight modifications in amino acid sequence (up to about two exchanges, additions, deletions) as long as the pharmaceutical properties of EPO in the ligated molecule are not adversely affected and as long as Asn24 is retained and Gly28 is retained at the C-terminus of the sequence of the fragment.

[0025] The term "C-terminal EPO fragment" or "EPO 29-165" refers to an EPO fragment having amino acids 29-165 of SEQ ID NO: 1 or of amino acids 29-166 of SEQ ID NO: 2. As mentioned above, the term also comprises fragments with slight modifications in amino acid sequence (up to about three exchanges, additions, deletions) as long as the pharmaceutical properties of EPO in the ligated mol-

ecule are not adversely affected and as long as Asn38 and Asn83 are retained and Cys29 is retained as N-terminal amino acid of said fragment.

[0026] According to the invention, the C-terminal part of erythropoietin beginning with Cys29 is produced recombinantly in eukaryotic cells, whereby Asn38 and Asn83 are glycosylated, while the N-terminal part from the beginning to Gly28 is synthesized in vitro by chemical reaction.

[0027] Therefore the invention provides a method for making an EPO mutant comprising chemically linking an N-terminal EPO fragment consisting of amino acids 1-28 of EPO having retained Asn24 and Gly28 with a C-terminal EPO fragment consisting of amino acids 29-165 or 166 of EPO being glycosylated at Asn38 and Asn83 and having retained Cys29 between Gly28 and Cys 29, and then isolating the EPO mutant.

[0028] Recombinant C-terminal EPO fragment may be prepared via expression in eukaryotic cells, for example in CHO, BHK or HeLa cell lines by recombinant DNA technology or by endogenous gene activation, that is, the erythropoietin glycoprotein is expressed by endogenous gene activation. The preferred erythropoietin mutants according to the invention are based on the sequence of human EPO. More preferably, the human EPO has the amino acid sequence set out in SEQ ID NO: 1 or SEQ ID NO: 2, most preferably, the human EPO having the amino acid sequence set out in SEQ ID NO: 1.

[0029] The C-terminal EPO fragment is produced preferably in CHO cells in the same manner as full-length EPO is produced, or preferably also by endogenous gene activation of human cells according to WO 99/05268 and EP 1 037 821.

[0030] The N-terminal EPO fragment can be synthesized by using stepwise solid-phase methods, cleaved from the resin and deprotected. It can be purified by chromatography, e.g., by high-performance liquid chromatography and characterized by ion-spray mass spectrometry as described, e.g., in Schnoelzer, M., et al., *Int. J. Pept. Protein Res.* 40 (1992) 180-193; Dawson, P. E., et al., *Science* 266 (1994) 776-779; and Schnoelzer et al., G. G. Fields (ed.), *Solid Phase Peptide Synthesis, Methods Enzymology* 289 (1977), see whole volume. At the C-terminus, the fragment preferably contains a thioester group for binding with Cys29 of the C-terminal fragment.

[0031] The ligation of the N-terminal and C-terminal EPO fragments can be performed by native chemical ligation in a simple manner as long as the C-terminal fragment has an amino-terminal cysteine residue, which preferably is Cys29 (Dawson, P. E., et al., *Science* 266 (1994) 776-779). According to this principle, the N-terminal fragment contains a thioester at the α -carboxyl group and undergoes nucleophilic attack by the side-chain of the cysteine residue at the amino terminus of the C-terminal fragment. The initial thioester ligation product undergoes rapid intramolecular reaction yielding a product with a native peptide bond at the ligation site. Such methods for chemical ligation of peptides are reviewed in Dawson, P. E., and Kent, S. B. H., *Annual Review of Biochemistry* 69 (2000) 923-960.

[0032] The N-terminal EPO fragment and the C-terminal EPO fragment were preferably solubilized during ligation in a denaturing solution such as guanidine hydrochloride or

urea and ligated according to Dawson, P. E., et al., *Science* 266 (1994) 776-779, or Dawson, P. E., et al., *J. Am. Chem. Soc.*, 119 (1997) 4325-4329.

[0033] The purification of the ligated EPO mutant is performed by conventional methods such as affinity chromatography, size-exclusion chromatography and ion-exchange chromatography.

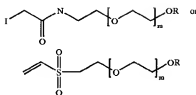
[0034] The term "PEGylation" means a covalent linkage of a (polyethylene) glycol residue at the N-terminus of the polypeptide and/or lysine 20. PEGylation of proteins is widely known in the state of the art and reviewed by, for example, Veronese, F. M., *Biomaterials* 22 (2001) 405-417. PEG can be linked using different functional groups and polyethylene glycols with different molecular weight, linear and branched PEGs as well as different linking groups (see also Francis, G. E., et al., *Int. J. Hematol.* 68 (1998) 1-18; Delgado, C., et al., *Crit. Rev. Ther. Drug Carrier Systems* 9 (1992) 249-304).

[0035] PEGylation of the N-terminal fragment can be performed in aqueous solution with PEGylation reagents as described, for example, in WO 00/44785, preferably using NHS-activated linear or branched PEG molecules of a molecular weight between 5 and 40 kDa. PEGylation can also be performed at the solid phase according to Lu, Y., et al., *Reactive Polymers* 22 (1994) 221-229.

[0036] These methods result in an N-terminal EPO fragment which is PEGylated at the ϵ -amino group of Lys20 and/or at the N-terminal amino group. Selective PEGylation at the N-terminal amino acid can be performed according to Felix, A. M., et al., *ACS Symp. Ser.* 680 (Poly(ethylene glycol)) (1997) 218-238. Selective N-terminal PEGylation are achieved during solid-phase synthesis by coupling of a N^o-PEGylated amino acid derivative to the N-1 terminal amino acid of the peptide chain. Side chain PEGylation are performed during solid-phase synthesis by coupling of N^o-PEGylated lysine derivatives to the growing chain. Combined N-terminal and side chain PEGylation are proceeded either as described above within solid-phase synthesis or by solution phase synthesis by applying activated PEG reagents to the amino deprotected peptide.

[0037] Suitable PEG derivatives are activated PEG molecules with a preferred average molecular weight of from about 5 to about 40 kDa, more preferably from about 20 to about 40 kDa, and most preferably about 30 kDa. The PEG derivatives can be linear or branched PEGs. A wide variety of PEG derivatives suitable for use in the preparation of PEG-protein and PEG-peptide conjugates can be obtained from Shearwater Polymers (Huntsville, Ala., U.S.A.; www.swpolymcrs.com).

[0038] Activated PEG derivatives are known in the art and are described in, for example, Morpurgo, M., et al., *J. Bioconjug. Chem.* 7 (1996) 363-368, for PEG-vinylsulfone. Linear chain and branched chain PEG species are suitable for the preparation of the PEGylated fragments. Examples of reactive PEG reagents are iodo-acetyl-methoxy-PEG and methoxy-PEG-vinylsulfone (m is preferably an integer from about 450 to about 900 and R is lower alkyl, linear or branched, having one to six carbon atoms such as methyl, ethyl, isopropyl, etc. whereby methyl is preferred):



[0039] The use of these iodo-activated substances is known in the art and described, e.g., by Hermanson, G. T., in *Bioconjugate Techniques*, Academic Press, San Diego (1996) p. 147-148.

[0040] Most preferably, the PEG species are activated by N-hydroxy succinimide ester using (alkoxy-PEG-N-hydroxysuccinimide, such as methoxy-PEG-N-hydroxysuccinimide (MW 30000; Shearwater Polymers, Inc.)), wherein R and m are as defined above.

[0041] The term "lower alkoxy" refers to an alkyl ether group in which the term "alkyl" means a straight-chain or branched-chain alkyl group containing a maximum of 4 carbon atoms, such as methoxy, ethoxy, n-propoxy and the like, and preferably methoxy.

[0042] The further purification of the PEGylated N-terminal fragments, including the separation of mono- or di-PEGylated species, are performed by methods known in the art, e.g., column chromatography.

[0043] Further, the present invention refers to an aqueous composition and a pharmaceutical composition comprising an EPO mutin or a composition as defined above and a pharmaceutically acceptable excipient, such as a buffer, and to the use of an EPO mutin or a composition as defined above for the preparation of pharmaceutical compositions for the treatment or prophylaxis of diseases correlated with anemia in chronic renal failure patients (CRF), AIDS and for the treatment of cancer patients undergoing chemotherapy. In addition, the invention refers to a method for the prophylactic and/or therapeutic treatment of disorders involving anemia in chronic renal failure patients (CRF), AIDS and cancer patients undergoing chemotherapy comprising the step of administering to a patient a composition as defined above in a therapeutically effective amount.

[0044] The term "therapeutically effective amount" is that amount of the erythropoietin mutin according to the invention necessary for the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. The exact amount of erythropoietin mutin is a matter of preference subject to such factors as the exact type of condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition. The pharmaceutical compositions containing the erythropoietin mutins are formulated at a strength effective for administration by various means to a human patient experiencing blood disorders characterized by low or defective red blood cell production. Average therapeutically effective amounts of the erythropoietin glycoprotein product may vary and in particular should be based upon the recommendations and prescription of a qualified physician.

[0045] Further, the invention refers to EPO mutins and compositions as defined above whenever prepared by the

processes described above and to EPO mutins and compositions as defined above for the treatment of diseases which are associated with anemia in chronic renal failure patients (CRF), AIDS and cancer patients undergoing chemotherapy.

[0046] The EPO fragments and EPO mutins according to the invention can be purified according to processes known in the art.

[0047] In EP-A 0 267 678, an ion exchange chromatography on S-Sepharose, a preparative reverse phase HPLC on a C8 column and a gel filtration chromatography are described for the purification of EPO. In this connection, the gel filtration chromatography step are replaced by ion exchange chromatography on S-Sepharose fast flow. It is also proposed that a dye chromatography on a Blue Trisacryl column be carried out before the ion exchange chromatography. A process for the purification of recombinant EPO is also described by Nobuo, I., et al., *J. Biochem.* 107 (1990) 352-359. In this process, EPO is treated however with a solution of Tween® 20, phenylmethylsulfonyl fluoride, ethylmaleimide, pepstatin A, copper sulfate and oxamic acid prior to the purification steps.

[0048] The specific activity of EPO or EPO mutins in accordance with this invention can be determined by various assays known in the art. The biological activity of the purified EPO proteins of this invention are such that administration of the EPO protein by injection to human patients results in bone marrow cells increasing production of reticulocytes and red blood cells compared to non-injected or control groups of subjects. The biological activity of the EPO mutins, or fragments thereof, obtained and purified in accordance with this invention can be tested by methods according to Pharm. Europa Spec. Issue Erythropoietin BRP Rio 1997(2).

[0049] Another biological assay for determining the activity of EPO, the normocythaemic mouse assay, is described in Example 5.

[0050] The erythropoietin mutin prepared in accordance with this invention may be prepared in pharmaceutical compositions suitable for injection with a pharmaceutically acceptable carrier or vehicle by methods known in the art. For example, appropriate compositions have been described in WO 97/09996, WO 97/40850, WO 98/58660, and WO 99/07401. Among the preferred pharmaceutically acceptable carriers for formulating the products of the invention include human serum albumin and human plasma proteins. The compounds of the present invention may be formulated in 10 mM sodium/potassium phosphate buffer at pH 7 containing a tonicity agent, e.g., 132 mM sodium chloride. Optionally, the pharmaceutical composition may contain a preservative. The pharmaceutical composition may contain different amounts of erythropoietin, e.g., 10 to 1000 µg/ml, e.g., 50 µg to 400 µg.

[0051] Administration of the erythropoietin glycoprotein products of the present invention results in red blood cell formation in humans. Therefore, administration of the erythropoietin glycoprotein products replenishes this EPO protein, which is important for the production of red blood cells. The pharmaceutical compositions containing the erythropoietin glycoprotein products may be formulated at a strength effective for administration by various means to a human

patient experiencing blood disorders, characterized by low or defective red blood cell production, either alone or as part condition or disease. The pharmaceutical compositions may be administered by injection such as by subcutaneous or intravenous injection. Average quantities of the erythropoietin glycoprotein product may vary and in particular should be based upon the recommendations and prescription of a qualified physician. The exact amount of conjugate is a matter of preference subject to such factors as the exact type of condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition. For example, 0.01 to 10 μg per kg body weight, preferably 0.1 to 1 μg per kg body weight, may be administered on a regular basis, e.g., once weekly.

[0052] Throughout this application, various publications have been referenced. The disclosures in these publications are incorporated herein by reference in order to describe more fully the state of the art.

[0053] The following examples, references and sequence listing are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

EXAMPLE 1

Production of PEGylated EPO (1-28): Synthesis of N^{α} -PEG- $\text{CH}_2\text{—CO—(Lys(PEG-CH}_2\text{—CO})^20\text{)}$ -EPO(1-28) α COSBzL and Isolation of Mono-PEGylated Fractions

[0054] Peptide was synthesized by using stepwise solid-phase methods, cleaved from the resin and deprotected, purified by high performance liquid chromatography and characterized by ion-spray mass spectrometry as described in literature (see Schnolzer, M., et al., *Int. J. Pept. Protein Res.* 40 (1992) 180; Schnölzer, P. M., et al., G. G. Fields, ed., *Solid-Phase Peptide Synthesis*, Meth. Enzymol. 289 (1997), whole volume), wherein for the formation of the C-terminal benzyl thioester the route of Lu, W., et al., *J. Am. Chem. Soc.*, 118 (1996) 8518–8523 was followed.

[0055] 1.1 Synthesis of PEG- $\text{CH}_2\text{—CO—NHS}$

[0056] mPEG- $\text{CH}_2\text{—COOH—(CH}_2\text{—(O—CH}_2\text{—CH}_2\text{—O—CH}_2\text{—COOH))}$ was activated with N-hydroxy-succinimide as described by Lu, Y. A., *Int. J. Pept. Protein Res.* 43 (1994) 127–138. Ethylenoxide repeating units were in the range of $n=110$ to give a molecular weight of 5000 (PEG₅₀₀₀), $n=440$ (PEG₄₀₀₀) or $n=880$ (PEG₈₀₀₀).

[0057] 1.2 Synthesis of EPO(1-28) α COSBzL

[0058] The N-terminal peptide was prepared with stepwise solid-phase synthesis according to Lu, W., et al., *J. Am. Chem. Soc.* 118 (1996) 8518–8523. BOC-Gly-(thioester linker)-amino methyl-resin was used for stepwise attachment of amino acids. The received peptide was deprotected and cleaved from the resin. Benzyl bromide was added to prepare the thioester which was then purified by HPLC. Thioester containing fractions were pooled and purified by HPLC.

[0059] 1.3 PEGylation of EPO(1-28) α COSBzL

[0060] PEG- $\text{CH}_2\text{—CO—NHS}$ was added to N-acylate the peptide at the $\alpha\text{-NH}_2$ of N-terminal alanine and at the $\epsilon\text{-NH}_2$ of lysine at position 20. The mono- and di-PEGylated EPO (1-28) thioesters were separated and purified by HPLC and lyophilized.

EXAMPLE 2

PEGylation of EPO (1-28) with Bifunctional Reagents

[0061] a) Covalent Linking of Thiol Groups

[0062] This example discloses the process in determining the reaction conditions for the covalent linking of thiol groups to the fragment. To determine the conditions, different amounts of a reagent containing a blocked thiol group. In this case, SATA (succinimidyl acetylthioacetate) or SATP (succinimidyl acetylthiopropionate) (dissolved in DMSO at 10 mg/ml) was added to a solution of the fragment EPO(1-28) α COSBzL, and then to 1 ml of 5 mg/ml fragment in 10 mM potassium phosphate, 50 mM NaCl, pH 7.3. The reaction was stirred for about 30 minutes (25° C.) and stopped by addition of 1 M lysine solution at 10 mM. Excess amounts of SATA and/or SATP were removed by dialysis against 10 mM potassium phosphate, 50 mM NaCl and 2 mM EDTA, pH 6.2. After removal of the protecting acetyl group with hydroxylamine, the number of thiol groups covalently linked to the fragment was determined photochemically with dithiodipyridine according to the method described by Grassetti, D. R. and Murray, J. F., in *J. Appl. Biochem. Biotechnol.* 119 (1967) 41–49.

[0063] b) PEGylation of Activated EPO (1-28)

[0064] 380 mg methoxy-PEG-maleimide (MW 30,000; Shearwater Polymers, Inc., Huntsville (Ala., USA)) was dissolved in a solution containing 95 mg activated EPO (4.5 mg/ml in 10 mM potassium phosphate, 50 mM NaCl, 2 mM EDTA, pH 6.2). The resulting molar ratio between activated fragment and methoxy-PEG-maleimide in the solution was 1:4. By addition of 1 M aqueous hydroxylamine solution at 30 mM, pH 6.2 to the above solution, the covalently linked blocked thiol groups of activated fragment were de-blocked. The resulting activated fragment in the reaction mixture of the solution contained free thiol (—SH) groups. De-blocking of the thiol groups was followed immediately by the coupling reaction between the activated fragment now containing free thiol (—SH) groups and methoxy-PEG-maleimide for 90 minutes (stirring, 25° C.). The coupling reaction was stopped by addition of 0.2 M aqueous cysteine solution at 2 mM to the reaction mixture. After 30 minutes excess free thiol groups of the activated fragment which did not react with methoxy-PEG-maleimide were blocked by addition of a 0.5 M N-methylmaleimide solution in DMSO to reach a concentration of 5 mM. After 30 minutes, the resulting reaction mixture now containing PEGylated fragment was purified by ion exchange chromatography and dialyzed against 10 mM potassium phosphate, pH 7.5 for ≥ 15 hours.

EXAMPLE 3

Recombinant Production of EPO (29-165)

[0065] EPO (29-165) was prepared in accordance with Example 1 of WO 99/05268.

[0066] Harvesting and Cell Separation

[0067] A batch refeed process was used, i.e., when the desired cell density was reached, approx. 80% of the culture was harvested. The remaining culture was replenished with fresh culture medium and cultivated until the next harvest. One production run consists of a maximum of 10 subsequent harvests: 9 partial harvests and 1 overall harvest at the end of fermentation. Harvesting takes place every 3-4 days.

[0068] The harvest volume was transferred into a cooled vessel. The cells were removed by centrifugation or filtration and discarded. The fragment containing supernatant of the centrifugation step was in-line filtered and collected in a second cooled vessel. Each harvest was processed separately during purification.

EXAMPLE 4

Ligation and Isolation

[0069] Equimolar amounts of both PEG-EPO (1-28) COS-Bzl prepared according to Examples 1 and 2 and EPO (29-165) were solubilized at about 6 mg/ml in 0.1 M phosphate buffer, 6 M guanidine-HCl at pH 7.5. 3% thiophenol and 1% benzyl mercaptan (by vol.) were added, as well as excess DTT to keep the protein reduced and ligated for about 36 hours until completion. Excess reagents were then

removed by ion exchange chromatography. Protein refolding was achieved by adjusting the buffer and pH to about 1 mg/ml protein concentration with 6 M guanidine HCl and dilution to about 0.2 mg/ml. The ligation product was purified according to Example 1 of WO 01/02017.

EXAMPLE 5

In Vivo Activity of PEGylated EPO Determined by the Normocythaemic Mouse Assay

[0070] PEG-EPO, unmodified EPO and buffer solution were administered to mice. The results show the superior activity and the prolonged half life of the PEGylated EPO species in regard to unmodified EPO indicated by the significantly increased amounts of reticulocytes and the shift of the reticulocytes count maximum using the same dose per mouse.

[0071] The normocythaemic mouse bioassay is known in the art (Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2)) and a method in the monography of erythropoietin of Ph. Eur. BRP. The samples were diluted with BSA-PBS. Normal healthy mice, 7-15 weeks old, were administered s.c. 0.2 ml of PEGylated EPO as described in Example 4. Over a period of 4 days starting 72 hours after the administration, blood was drawn by puncture of the tail vein and diluted such that 1 μ l of blood was present in 1 ml of an 0.15 μ mol acridine orange staining solution. The staining time was 3 to 10 minutes. The reticulocyte counts were carried out microfluorometrically in a flow cytometer by analysis of the red fluorescence histogram (per 30,000 blood cells analyzed). Each investigated group consisted of 5 mice per day, and the mice were bled only once.

SEQUENCE LISTING

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<210> SEQ ID NO 1

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          20           25           30
Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
          35           40           45
Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
          50           55           60
Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
          65           70           75           80
Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
          85           90           95
Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Ala Leu
          100          105          110
Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
          115          120          125

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-continued

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Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
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Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala
145                150                155                160

Cys Arg Thr Gly Asp
      165

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 1          5          10          15

Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His
      20          25          30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe
      35          40          45

Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp
      50          55          60

Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu
      65          70          75          80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp
      85          90          95

Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu
      100         105         110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala
      115         120         125

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val
      130         135         140

Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala
      145         150         155         160

Cys Arg Thr Gly Arg
      165

```

1. An erythropoietin mutein comprising N-glycosylated sites at Asn38 and Asn83 but not at Asn24.

2. The erythropoietin mutein according to claim 1, comprising linking the N-terminal amino group to lower alkoxy-poly(ethylene glycol) group(s).

3. The erythropoietin mutein according to claim 1, comprising linking the ϵ -amino group of Lys20 to lower alkoxy-poly(ethylene glycol) groups.

4. The erythropoietin mutein according to claim 2, wherein the average molecular weight of each poly(ethylene glycol) moiety is from about 5 kilodaltons to about 40 kilodaltons.

5. The erythropoietin mutein according to claim 3, wherein the average molecular weight of each poly(ethylene glycol) moiety is from about 5 kilodaltons to about 40 kilodaltons.

6. The erythropoietin mutein according to claim 2, wherein the average molecular weight of each poly(ethylene glycol) moiety is about 30 kilodaltons.

7. The erythropoietin mutein according to claim 2, wherein the average molecular weight of each poly(ethylene glycol) moiety is about 30 kilodaltons.

8. The erythropoietin mutein of claim 2 further comprising linking the ϵ -amino group of Lys20 to lower alkoxy-poly(ethylene glycol) groups.

9. The erythropoietin mutein according to claim 2, wherein the poly(ethylene glycol) moieties are capped by a methoxy group.

10. The erythropoietin mutein of claim 3, wherein the poly(ethylene glycol) moieties are capped by a methoxy group.

11. The erythropoietin mutein of claim 1, comprising the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

12. An aqueous composition comprising an erythropoietin mutein having N-glycosylation sites at Asn38 and Asn83 but not at Asn24, and a pharmaceutically acceptable buffer.

13. The aqueous composition according to claim 12, comprising linking the erythropoietin mutein at the N-terminal amino group to lower alkoxy poly(ethylene glycol) group(s).

14. The aqueous composition according to claim 12, comprising linking the erythropoietin mutein at the ε-amino group of Lys20 to lower alkoxy poly(ethylene glycol) group(s).

15. A pharmaceutical composition comprising a mutein according to claim 1 and a pharmaceutical acceptable carrier.

16. A composition comprising an aqueous composition according to claim 12 and a pharmaceutical acceptable carrier.

17. A method of treating a disease relating to anemia in chronic renal failure patients comprising administering to a patient in need thereof a therapeutically effective amount of an erythropoietin mutein according to claim 1.

18. A method of treating a disease relating to anemia in chronic renal failure patients comprising administering to a patient in need thereof a therapeutically effective amount of a composition according to claim 12.

19. A method of treating anemia in AIDS patients and cancer patients undergoing chemotherapy comprising administering to a patient in need thereof a therapeutically effective amount of an erythropoietin mutein according to claim 1.

20. A method of treating anemia in AIDS patients and cancer patients undergoing chemotherapy comprising

administering to a patient in need thereof a therapeutically effective amount of a composition according to claim 12.

21. A method of making an erythropoietin mutein, comprising chemically linking an N-terminal erythropoietin fragment consisting of amino acids 1-28 of erythropoietin having retained Asn24 and Gly28 with a C-terminal erythropoietin fragment consisting of amino acids 29-165 or 29-166 of erythropoietin being glycosylated at Asn38 and Asn83, and having retained Cys29, said linkage occurring between Gly28 and Cys 29, and isolating the erythropoietin mutein.

22. The method according to claim 21, comprising covalently linking a polyethylene glycol residue of the N-terminal erythropoietin fragment at the N-terminus of the erythropoietin sequence.

23. The method according to claim 21, comprising covalently linking a polyethylene glycol residue of N-terminal erythropoietin fragment at the lysine 20 of the erythropoietin sequence.

24. The method according to claim 21, comprising producing the N-terminal fragment by chemical synthesis.

25. The method according to claim 21, comprising producing the C-terminal fragment by recombinant gene expression.

* * * * *

Structural and functional characterisation of recombinant human erythropoietin analogues

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Received 22 October 1993

Deletion mutants of a synthetic human erythropoietin-cDNA were transiently expressed in COS-7 cells and products analyzed using Western blots and a cell proliferation assay. Only two mutants with deletions affecting the N-terminal portion and the amino acid sequence 115-121 displayed biological activity. The exchange of hydrophilic, charged amino acids by alanine in two potential α -helical regions, the internal amino acid sequence 102-106 and the C-terminal sequence 154-159, causes a 2-11-fold loss of activity. The results suggest that both regions are involved in either maintaining the active structure of the hormone or interacting with the receptor.

Erythropoietin; Mutagenesis; Transient expression; Structure-function relationship

1. INTRODUCTION

The proliferation and differentiation of erythroid progenitor cells are regulated by the glycoprotein hormone erythropoietin [1]. Although the recombinant protein is available and in clinical use for some years many questions concerning the molecular mechanism of EPO's action remain to be answered. Further understanding of its function may be gained from the analysis of the interaction with the EPO-receptor and the signal transduction pathways induced.

A number of studies have shown that the glycosylation, which accounts for 40% of the molecular mass, is responsible for the *in vivo* stability, cellular processing and secretion [2,3,4,5]. The protein portion of the molecule is essential for interaction with the receptor. Information available on the location of the receptor binding site has been generated by strategies involving anti-peptide antibodies [6,7,8], mutational analysis [9,10,11] and by comparing the sequences of EPO genes from different species [12]. Extensive studies with anti-peptide antibodies show that only those which are derived from peptides covering an internal region (amino acids 99-119; 111-129) and the C-terminal portion (amino acids 152-166) are able to neutralize the biological activity of EPO [7,8]. Deletion of amino acids in regions predicted to have a α -helical structure result in the expression of proteins which are not exported by the cells, whereas mutations within the interhelical loops do not affect the

export and biological activity of the products in most cases [9,10,11]. A study on the region between amino acids 99-129 provides evidence that changes to residues 99-110 lead to an inactivation of EPO [11].

We have further characterized the role of regions predicted to be functionally important and shown that deletions, even single amino acid substitutions in the C-terminus and residues 102-106, lead to a loss or significant reduction of the biological activity suggesting that these regions are directly involved in the binding of EPO to its receptor. Moreover, our results exclude an N-terminal region and the amino acids 115-121 as functionally active portions of the molecule.

2. MATERIAL AND METHODS

2.1. EPO gene synthesis

A synthetic human EPO gene was generated by a chemoenzymatic strategy.

The amino acid sequence was transferred into a DNA-sequence by a computer program creating as many restriction sites as possible without changing the amino acid composition. The coding region was assembled by the sequential ligation of eight blocks consisting of a pair of oligonucleotides each. The oligonucleotides were designed to have complementary regions at their 3' termini and compatible restriction sites to join the blocks. After annealing the oligonucleotide pairs they were filled in with Klenow-polymerase to give full-length double stranded products. Individual blocks were cloned, sequenced and ligated using standard procedures [13].

2.2. Deletion mutagenesis and site-directed mutagenesis

The gene synthesis strategy used provided enough possibilities for deletions in the entire coding region. The deletions were introduced using restriction enzymes. The products were subcloned into the plasmid puc19. Site-directed mutagenesis was carried out with a Mutagenesis M13 *in vitro* Mutagenesis Kit (Biorad, Richmond, CA) according to a method published by Kunkel [14]. The presence of all mutations was confirmed by sequencing all constructs with the dideoxy

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Abbreviations: EPO, erythropoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor.

chain termination method [15] and a sequencing kit supplied by USB (Cleveland, OH).

2.3. Expression vector constructs

The human EPO mutants were subcloned into the *EcoRI* restriction site of the plasmid pSVSPORT1 (Gibco, BRL) containing the SV40 origin of replication, early transcriptional promoter, small t-intron and polyadenylation signals (Fig. 1). The orientation of the inserts was confirmed by restriction-mapping. Plasmid DNA was prepared by CsCl-gradient centrifugation followed by two extractions with water-saturated isomylalcohol.

2.4. Transfection of EPO and its analogues

The DNA constructs were transfected into COS-7 cells. COS-7 cells were grown to 80% confluence in Dulbecco's Modified Eagle's medium (DMEM) containing 20 mM HEPES and supplemented with 10% fetal calf serum (FCS). Usually, 20 µg CsCl-purified non-linearized plasmid-DNA and 20 µg sonicated salmon sperm DNA were combined with 4×10^6 cells in 800 µl medium supplemented with 10% FCS. The electroporation was performed with a BioRadGenePulser [16]. A capacitance of 960 µF and a voltage of 250 V were found to produce the highest transfection efficiency. Transfections with the vector pSVSPORT1 were included as controls. The single cell suspension was plated in growth medium immediately post transfection. After 24 h of growth as adherent monolayers the medium was replaced by FCS-free medium and the cells incubated for another 24 h. The conditioned medium was harvested and used for the EPO assays.

2.5. Detection of mutant EPO proteins

EPO-protein was detected by Western blots using a monoclonal antibody directed against the NH₂-terminal portion of the amino acid sequence supplied by Medac (Hamburg). EPO-containing COS-cell supernatants were size-fractionated by SDS-PAGE using the buffer system described by Laemmli [17] and 10% gels. For Western blot analysis, proteins were transferred to a 0.2 mm membrane (Hybond-ECL, Amersham) by semi-dry blotting. Non-specific binding sites on the filter were blocked by incubation in PBS/0.1% Tween (PBS-T). Blots were incubated with the anti-EPO-antibody (10 µg) in PBS-T containing 1% BSA for 2 h. After washing with PBS-T and incubation with a horseradish peroxidase labelled anti-mouse Ig antibody (Amersham, 1:5000 in PBS-T/1% BSA) for one hour, the blots were washed with PBS and developed with the ECL-system purchased from Amersham. The bands were scanned by an imaging densitometer (GS-670, Biorad). Several exposures were taken to ensure that the signals were in the linear range of film response. The quantification of EPO-protein was performed by the comparative analysis with pure recombinant EPO.

2.6. Stimulation of hematopoietic cell proliferation

The biological activity of EPO mutant proteins was measured by an *in vitro* bioassay using the EPO-sensitive cell line TF-1. This human erythroleukemic cell line was established by Kitamura et al. [18] and kindly supplied by W. Osterst, Heinrich-Pette-Institut, Hamburg. The cells were cultivated in RPMI 1640 medium (Gibco) supplemented with 10% FCS and 7.5 ng/ml GM-CSF. Exponentially growing cells were washed free of growth factor, maintained 24 h without GM-CSF and exposed to dilutions of the conditioned COS-cell supernatants for an additional 48 h. The proliferation of the cells was measured with the MTT reduction assay essentially as described by Mosmann [19]. The activity of the mutant proteins was determined after Western blot quantification using recombinant human EPO with known specific activity as a standard.

3. RESULTS

A synthetic EPO-cDNA was constructed by a chemoenzymatic approach. The strategy involved the introduction of additional restriction sites, which were used

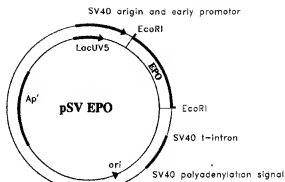


Fig. 1. Expression vector pSV EPO.

to generate a series of deletion mutants. Fig. 2 depicts the structure of the mature human EPO-molecule and shows the location of the deletions and the amino acid substitutions as well as the predicted α -helices in its secondary structure.

The EPO-analogues were designed with regard to earlier reports of mutational analysis [10,11], experiments with anti-peptide antibodies [6,7,8,24] and evolutionary considerations [12].

The amino acids deleted or changed are shown in Table I. Besides the deletion in EPO^d all mutations are located within one of the four predicted α -helices. These α -helices are thought to be critical for the tertiary structure of growth factors and hence may represent the receptor-binding portion of several hematopoietic growth factors, hormones and cytokines [20]. The deletion of the EPO^d mutant is located in the loop joining α -helices C and D. Peptides covering this region were shown to induce antibodies which are able to neutralize EPO's activity [7].

After subcloning the constructs into the expression vector shown in Fig. 1 the cDNA's were transiently expressed in COS-7 cells. By analyzing the supernatants

Table I
Erythropoietin analogues created by deletion and site-directed mutagenesis

Construct	Amino acids deleted or changed	α -Helix position*
EPO ^{WT}	—	—
EPO ^a	Gln ¹³ -Arg ¹⁶ -Tyr ¹³ -Leu ¹⁶ -Leu ¹⁷	A
EPO ^b	Gln ¹³ -Gly ¹⁶ -Leu ¹³ -Ala ¹⁶ -Leu ¹⁷	B
EPO ^c	Leu ¹³ -Arg ¹⁶ -Ser ¹⁶ -Leu ¹³ -Thr ¹⁶	C
EPO ^d	Glu ¹³ -Lys ¹⁶ -Glu ¹³ -Ala ¹⁶ -Ile ¹³ -Ser ¹⁶	—
	Pro ¹⁷	—
EPO ^e	Lys ¹³ -Leu ¹³ -Tyr ¹⁶ -Thr ¹³ -Gly ¹³ -Gly ¹³	D
EPO ¹⁸	Arg ¹³ → Ala ¹³	C
EPO ¹⁹	Thr ¹⁶ → Ala ¹⁶	C
EPO ¹⁴	Lys ¹³ → Ala ¹³	D
EPO ¹⁵	Glu ¹³ → Ala ¹³	D

*Predicted by Buzan [20], see Fig. 2.

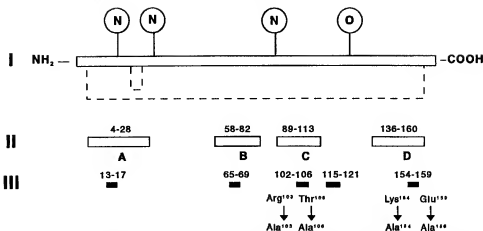


Fig. 2. Structural features of the human EPO-molecule and location of the investigated deletions/amino acid substitutions. (I) Primary structure of the mature human EPO-molecule comprising 165 amino acids; N- and O-linked glycosylation sites are shown by circles; the dashed lines represent the disulphide bonds. (II) Rectangles show the location of the investigated deletions/amino acid substitutions that were introduced into wild-type-EPO (WT-EPO).

in Western blots we could show that all the recombinant proteins except EPO³ were synthesized by the COS cells (Fig. 3). In the case of EPO³ (deletion of amino acids 102–106), protein was not detectable in lysed cells (results not shown) as well as supernatants. In comparison, Chern et al. [11] report the inability of COS cells to synthesize detectable amounts of EPO after transfection with mutants affecting the amino acids 99–108. The quantification of the Western blot signals with the help of a standard and densitometric scanning revealed secretion levels in the range of 15–24 ng/ml. The molecular weight of the proteins was in the expected range with a very similar molecular weight heterogeneity, indicating a normal post-translational modification of the products. The supernatants were assayed for their ability to stimulate growth of the EPO-responsive cell line TF-1, as well as their content of immunoreactive protein. The results presented in Table II, indicate that the wild-type cDNA was efficiently expressed and secreted. Only two of five EPO-analogues with amino acid deletions, EPO⁴ (N-terminal amino acids 13–17) and EPO⁴ (internal amino acids 115–121), were found to be active. The deletions of C-terminal amino acids 154–159

(EPO⁵) and two internal sequences (amino acids 65–69/EPO⁶; amino acids 102–106/EPO³) are obviously deleterious for the expression of an active protein. However, the amino acid substitutions affecting the same α -helical regions as the deletions in EPO³ and EPO⁴, resulted in detectable, although significantly reduced, activity. The exchange of the hydrophilic amino acids residues Arg¹⁰³, Thr¹⁰⁶, Lys¹⁵⁴ and Glu¹⁵⁹ with the neutral amino acid alanine causes a 2–11-fold loss of relative specific activity.

4. DISCUSSION

The aim of this study was to characterize regions of the EPO-molecule which are thought to participate in the interaction with the EPO-receptor and to identify critical amino acid residues involved. In initial experiments internal deletions of 5–7 amino acids were introduced into the wild-type EPO to reveal functionally important domains. Four of the five deletion mutants were effectively expressed as shown by Western blot analysis, but only two of them displayed biological activity.

A mutant with a deletion located in the N-terminal part (EPO³) had nearly the same biological activity as the wild-type EPO, confirming the observation that antibodies specific for this region do not neutralize the activity of the hormone [6,21]. Another mutant (EPO⁴ lacking amino acids 115–121) with the deletion in the region which is predicted to join α -helices C and D [20] displayed slightly decreased activity. Similar results were shown by Boissel et al. [9] analyzing EPO-mutants with a deletion of the amino acids 111–119 or an insertion of a myc epitope after Lys¹¹⁶, and by Chern et al. [11] using sequential alteration of the secondary structure defined by the amino acids 120–129.

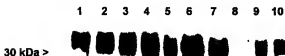


Fig. 3. Western blot analysis of recombinant human EPO-analogues in COS-7-cell supernatants. Recombinant EPO and the analogues were detected by Western blotting with an anti-EPO-antibody and display the normal size of fully glycosylated EPO. All COS-cell supernatants expressed the transfected EPO gene except EPO³. Lane 1 = EPO¹; lane 2 = EPO¹⁰; lane 3 = EPO¹⁰; lane 4 = EPO¹⁰; lane 5 = EPO¹⁰; lane 6 = EPO¹⁰; lane 7 = EPO¹⁰; lane 8 = EPO¹⁰; lane 9 = EPO¹⁰; lane 10 = EPO¹⁰.

In contrast, any deletions in this study and reported by others [9,10,11] affecting the α -helical stretches close to the C-terminus (helices C, D) were deleterious for hormonal activity, probably by causing misfolding of the products. The model for the tertiary structure of EPO and several other growth factors and hormones presented by Bazan [20] would suggest that the helical structure near the C-terminus comprises the receptor binding structure. Studies using antipeptide antibodies to hydrophilic regions show that antibodies to the C-terminal amino acids [8], as well as to different peptides covering the amino acids 99–129 [7], are able to neutralize the bioactivity of EPO. In addition, EPO molecules tagged with a hemagglutinin influenza virus epitope or a consensus sequence for phosphorylation at the C-terminus were inactive [22]. From all data available it is evident that the internal and C-terminal regions are necessary to maintain a biologically active structure and are possibly involved in receptor binding.

Deletions of amino acids, as well as the binding of antibodies can lead to gross structural alterations and consequently the inactivation of proteins. Therefore, single amino acid changes were introduced into the molecule in this study to determine if the exchange of residues which are known to be typically involved in receptor interactions, affected the bioactivity. The residues selected for mutagenesis are highly conserved in different EPO-species [12]. Amino acids with hydrophilic, charged side chains were replaced by the neutral amino acid alanine to minimize alterations in the backbone conformation of the recombinant EPO. The lysine residue at position 154 is predicted to be exposed on the surface of helix D which displays conserved sequence patterns in a number of cytokines [20,22]. In each case a loss of specific activity was observed, with the greatest

reduction in activity detected in the mutants affecting the region close to the C-terminus.

The data presented here, therefore, indicate the important role of the amino acid sequences 102–106 and 154–159 and define single residues within the helices C and D, which may be involved in the receptor interaction of EPO.

Acknowledgements: This work was supported by a grant from the Deutsche Forschungsgemeinschaft. Th.B. is a recipient of a research award of the Sandoz-Foundation.

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Table II

Biological activity of Erythropoietin in COS-7-cell supernatants

Construct	Biological activity (U/ml)*	Relative specific activity (%)**
EPO ^{WT}	3.7 ± 0.4	100
EPO ^A	3.4 ± 0.5	109
EPO ^B	n.d.	—
EPO ^C	n.d.	—
EPO ^D	2.5 ± 0.4	88
EPO ^E	n.d.	—
EPO ¹⁰³	1.2 ± 0.3	47
EPO ¹⁰⁶	1.6 ± 0.3	41
EPO ¹⁵⁴	0.4 ± 0.1	9
EPO ¹⁵⁹	0.8 ± 0.2	26

*Determined by a cell proliferation assay (see Section 2) data expressed as means ± S.D. (n = 3)

**Calculated by comparing the quotient of biological activity/immunoreactive protein content for each EPO analogue to the wild type EPO.

n.d. = not detectable.

The Protein Encoded by a Growth Arrest-Specific Gene (*gas6*) Is a New Member of the Vitamin K-Dependent Proteins Related to Protein S, a Negative Coregulator in the Blood Coagulation Cascade

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Received 12 April 1993/Returned for modification 27 April 1993/Accepted 19 May 1993

A set of growth arrest-specific genes (*gas*) whose expression is negatively regulated after serum induction has previously been described (C. Schneider, R. M. King, and L. Philipson, *Cell* 54:787-793, 1988). The detailed analysis of one of them, *gas6*, is reported here. *gas6* mRNA (2.6 kb) is abundantly expressed in serum-starved (48 h in 0.5% fetal calf serum) NIH 3T3 cells but decreases dramatically after fetal calf serum or basic fibroblast growth factor stimulation. The human homolog of *gas6* was also cloned and sequenced, revealing a high degree of homology and a similar pattern of expression in IMR90 human fibroblasts. Computer analysis of the protein encoded by murine and human *gas6* cDNAs showed significant homology (43 and 44% amino acid identity, respectively) to human protein S, a negative coregulator in the blood coagulation pathway. By using an anti-human *Gas6* monospecific affinity-purified antibody, we show that the biosynthetic level of human *Gas6* fully reflects mRNA expression in IMR90 human fibroblasts. This finding thus defines a new member of vitamin K-dependent proteins that is expressed in many human and mouse tissues and may be involved in the regulation of a protease cascade relevant in growth regulation.

Interactions between serine proteases, their substrates, and their inhibitors have largely been exploited during evolution. Protease cascades are not confined to the classical blood coagulation or complement cascade. A network of proteases that control the synthesis or activity of a ligand appears an ideal and finely regulatable mechanism to trigger a rapid response to an extracellular event, with the inherent advantage of powerful amplification. Thrombin, in addition to catalyzing fibrin polymerization, can act as a novel ligand for the recently identified thrombin receptor family, possibly mediating other known effects of thrombin, including its role as a mitogen for lymphocytes and fibroblasts (8, 9). Hepatocyte growth factor (scatter factor), which promotes cell division (53) and epithelial morphogenesis (47), is similar in structure to serine proteases (38% amino acid sequence identity with plasminogen), although it lacks proteolytic activity as a result of mutation of two residues in the catalytic triad (31, 48). Hepatocyte growth factor is the ligand for the *c-met* proto-oncogene product (5, 49), a transmembrane 190-kDa heterodimer with tyrosine kinase activity that is widely expressed in normal epithelial tissues (20).

Recently, a protease pathway has been shown to play a crucial role in the dorsoventral patterning of *Drosophila* embryos (36). At least three genes (*snake*, *gastrulation defective*, and *easter*) appear to encode extracellular proteases (7, 36). Easter appears to be the ultimate protease that processes spätzle that binds and activates its receptor Toll

(36). The ultimate function of this pathway is to promote the translocation of dorsal protein to the nucleus, where it regulates the transcription of a number of target genes, including *tolloid*. The product of *tolloid* contains a protease domain, homologous to human bone morphogenetic protein 1 (55), that is involved in the activation of decapentaplegic (*dpp*), a member of the transforming growth factor β 1 family (50).

In analogy to this finely dissected developmental system, a considerable body of evidence has pointed to a set of different proteases, including serine, cysteine, and metalloproteases, as prime candidates in the regulation of tumor invasion and angiogenesis (41, 46). The activities of these proteases are strictly regulated at the levels of both gene expression and zymogen activation (45). Furthermore, the activities of most of these proteases appear to be enhanced when the enzymes are cell membrane associated. Cell-bound proteases are subject to negative regulation by natural protease inhibitors (10). Although current knowledge of protease cascades relates to tissue remodeling during tumor invasion and angiogenesis, other cells must perform similar functions. In fact, normal tissue homeostasis is dependent on balanced rates of cell division, extracellular matrix (ECM) synthesis, and degradation. Recent evidence has demonstrated a close link between cytokines and growth factors that directly modulate the three processes. The ECM acts as a reservoir for several growth factors and modulates their activities (26). There is evidence that a number of proteases is involved in growth factor mobilization from the ECM (3, 25).

To dissect the mechanism that controls growth arrest in mammalian cells, a set of genes that are highly expressed during serum starvation in NIH 3T3 mouse fibroblasts was

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previously cloned (54). In this report, we characterize a growth arrest-specific gene, *gas6*, whose expression is negatively regulated during growth induction. The product of *gas6* represents a new member of the vitamin K-dependent family that is homologous to protein S, a cofactor of the activated protein C pathway that leads to the proteolytic inactivation of factors Va and VIIIa and ultimately to an effective anticoagulant action (23, 24). This finding may thus uncover a general important contribution of growth-regulated protease cascades to cell proliferation.

MATERIALS AND METHODS

Cell lines and cell culture conditions. NIH 3T3 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml). For serum starvation, NIH 3T3 cells plated at $10^4/\text{cm}^2$ were shifted to 0.5% FCS for 48 h. Under these conditions, incubation with 50 µM bromodeoxyuridine (BrdU) for 3 h resulted in labeling of less than 2% of the nuclei. For induction of DNA synthesis, fresh medium containing 20% FCS was added to growth-arrested cells. Cells were harvested at various times for RNA isolation. After 18 h of BrdU incorporation, more than 90% of the nuclei scored positive for DNA synthesis. For density-dependent inhibition, cells were plated at $10^4/\text{cm}^2$ in 10% FCS. Twenty-four hours after plating, the medium was changed every 2 days. After 4 days in culture, incubation with BrdU for 2 h yielded less than 1% of labeled nuclei. DNA synthesis was determined with a mouse monoclonal antibody against BrdU as described elsewhere (6). Human IMR90 fibroblasts were obtained from the Genetic Mutant Repository (Camden, N.J.) and grown as recommended. For serum starvation, subconfluent cells were shifted to 0.5% FCS for 72 h. Under these conditions, incubation with BrdU for 3 h resulted in labeling of less than 3% of the nuclei. DNA synthesis was induced as described above; after 18 h of BrdU incorporation, more than 45% of the nuclei stained positive. Basic fibroblast growth factor (bFGF) was kindly supplied by C. Grassi-Farmitalia, Milano, Italy, and used at a concentration of 100 ng/ml.

RNA preparation and Northern (RNA) blotting analysis. For extraction of total RNA from cells, the cultures were washed twice with phosphate-buffered saline, and lysis buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.1 M 2-mercaptoethanol, 0.5% N-laurylsarcosine) was added. RNA from mouse or human tissues was extracted by disrupting the tissue in lysis buffer with a Polytron homogenizer. RNA was then isolated as described previously (11). Total RNA (20 µg) was separated on 1% agarose gels containing 6.7% formaldehyde (40) and transferred to Duralon-UV nylon membranes (Stratagene) by using a 2016 VacuGene apparatus (Pharmacia). RNA was cross-linked by exposure to UV light (Stratalinker; Stratagene). Hybridization was performed in 1 M NaCl–1% sodium dodecyl sulfate (SDS) at 65°C, using the corresponding probes labeled with ^{32}P by random-primer synthesis (Pharmacia).

Isolation of full-length cDNA clones for *gas6*. The original *gas6* clone (54) was used to screen two cDNA libraries generated from G₀ NIH 3T3 fibroblasts and mouse kidney mRNA (34) and cloned by an orientation-specific strategy (22) in the lambda vector T7-T3/E-H (33). The murine cDNA clone was used to screen a human lung fibroblast cDNA library (Stratagene) at low stringency, and a partial cDNA clone was isolated. To isolate the complete cDNA, a HeLa cDNA library generated in the lambda vector 1149 (53a) was

screened under high-stringency conditions, using the partial human *gas6* (h*gas6*) insert.

DNA sequencing and sequence analysis. All DNA fragments, obtained by using appropriate restriction enzymes, were subcloned in the Bluescript KS+ plasmid (Stratagene). Plasmid and lambda DNAs were isolated and sequenced (18) with the T7 sequencing Kit (Pharmacia). Specific synthetic oligonucleotides were also used as primers for the sequencing reactions. The sequence of each nucleotide was determined three times on average, and the entire sequence was read on both strands. The sequence of the h*gas6* cDNA clone was obtained by using the EMBL-ALF sequencer. Sequence analysis was performed by using the Intelligent-ics software package.

Polyclonal antibody preparation. The cDNA of h*gas6* was digested with *PvuII*, and the resulting fragment from nucleotides (nt) 1209 to 1788 was ligated to *Bam*HI adaptors and inserted into the *Bam*HI site of the pAR 3038 vector, which carries the promoter of the Ψ 10 gene of T7 bacteriophage (58). Expression of T7 RNA polymerase was performed by infection of host cells (*Escherichia coli* Q358) with bacteriophage λ -CEG, carrying the bacteriophage T7 gene, at a multiplicity of infection of 5 to 7. Protein expression and purification were performed as described previously (6). Rabbits were injected with 200 µg of purified bacterial h*Gas6* protein mixed with an equal volume of complete Freund's adjuvant. The animals were then injected with the same amount of protein in incomplete Freund's adjuvant every 3 weeks for 2 months. Specific antibodies were affinity purified by using 0.5 mg of h*Gas6* protein covalently coupled to Affi-Prep 10 (Bio-Rad Laboratories, Cambridge, Mass.) as described previously (6).

In vitro translation of h*gas6*. In vitro translatable h*gas6* RNA was generated from the pCITE-1 vector (Novagene, Madison, Wis.), which contains an RNA capping-independent translation enhancer sequence downstream of the T7 polymerase promoter. pCITE-h*gas6* contains a cDNA fragment from the ATG (nt 135) to the end of the clone and was cloned in pCITE in two steps. First, the h*gas6* cloned in pBluescript KS+ was digested with *NcoI*, which cuts twice in h*gas6* at position 134, corresponding to the initial methionine, and at position 1260; the resulting fragment was inserted in the same site of the pCITE vector. Second, h*gas6* pBluescript KS+ was digested with *SacI*, which cuts at nt 698 of the cDNA, and *SacI*, which is present in the polylinker of the plasmid 3' to the cDNA. The resulting fragment was inserted into pCITE/*NcoI*-containing fragment digested with the same enzymes. The pCITE-h*gas6* was then linearized with *SacI*, transcribed, and translated as described previously (43). For immunoprecipitation, 5 µl of the reticulocyte translation mixture was mixed with 0.1 ml of Nonidet P-40 (NP-40) buffer (50 mM triethanolamine [TEA; pH 7.5], 0.1% NP-40, 150 mM NaCl) and incubated for 1 h on ice with anti-h*Gas6* affinity-purified antibody; 50 µl of a 10% (wt/vol) suspension of protein A-Sepharose (Pharmacia Fine Chemicals) was added, and incubation was prolonged for 30 min at 4°C with rocking. After three washes with NP-40 buffer, the immunocomplex was resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gel was fixed in methanol-acetic acid and treated for fluorography with Enlightening (DuPont).

In vivo biosynthesis of h*Gas6*. Human IMR90 fibroblasts were labeled under different growth conditions for 3 h in 0.7 ml of methionine-free Dulbecco's modified Eagle medium containing [^{35}S]methionine (ICN-TRANS ^{35}S label; 1,133 Ci/mmol) at ~ 500 µCi/ml. At the end of the labeling period,

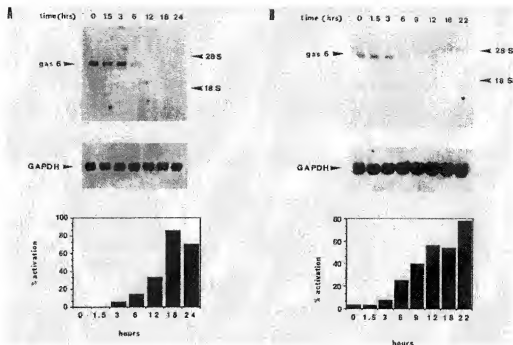


FIG. 1. Growth cycle regulation of *gas6* gene expression in NIH 3T3 cells. RNA was extracted from arrested NIH 3T3 cells (48 h in 0.5% FCS) and at the indicated times after addition of 20% FCS (A) or bFGF (B). Equal amounts (20 μ g) of total RNA were analyzed by Northern blotting. The same blots were also probed with a *gapdh* cDNA probe. The histograms show the relative level of DNA synthesis for each time point analyzed on the Northern blots.

the medium was collected and supplemented with 50 mM TEA (pH 7.4), 150 mM NaCl, and 0.8% SDS (final concentrations). The cell monolayer was lysed with 0.5 ml of lysis buffer (150 mM NaCl, 50 mM TEA [pH 7.5], 0.1% NP-40) on ice for 3 min and then added with 0.8% SDS (final concentration). Both cell lysate and culture supernatant were then boiled for 4 min. After boiling, an equal volume of SDS quench buffer (150 mM NaCl, 50 mM TEA [pH 7.5], 4% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μ g each of aprotinin, leupeptin, antipain, and pepstatin per ml) was added. After clearing by centrifugation at 12,000 rpm for 2 min, the supernatants were incubated with 30 μ l of normal rabbit serum for 1 h on ice. Samples were then transferred to an Eppendorf tube containing 20 μ l of pre-washed staphylococcal protein A and incubated at 4°C for 30 min with continuous rocking. After centrifugation for 2 min, the resulting supernatant was similarly treated once more and finally centrifuged for 5 min. At the end of these two pre-clearing steps, samples were immunoprecipitated by incubation with the affinity-purified anti-hGas6 antibody for 3 h at 4°C with rocking; 80 μ l of protein A-Sepharose (10% [wt/vol]) suspension was added, and incubation was continued for 30 min at 4°C with rocking. Protein A-Sepharose was recovered by centrifugation, washed three times with 0.5% Triton X-100–20 mM TEA–150 mM NaCl–1 mM phenylmethylsulfonyl fluoride, and resuspended in SDS sample buffer. Immune complexes were released by boiling for 5 min and analyzed by SDS-PAGE as described above.

Nucleotide sequence accession numbers. The nucleic acid sequences of the murine and human *gas6* cDNAs have been submitted to the EMBL, GenBank, and DDBJ data

banks under accession numbers X59846 and L13720, respectively.

RESULTS

Regulation of *gas6* mRNA expression by serum and bFGF at growth arrest and during the cell cycle. *gas6* belongs to a category of genes previously identified as growth arrest specific, because their expression is down-regulated after growth induction in arrested NIH 3T3 cells. Figure 1 shows a Northern blot analysis of *gas6* expression at various times after a synchronous cell division cycle induced either with FCS (Fig. 1A) or bFGF (Fig. 1B) in NIH 3T3 cells arrested for 48 h in 0.5% FCS (time 0). The mRNA identified by the *gas6* murine cDNA is about 2.6 kb in size and is abundantly expressed at growth arrest (time 0 in Fig. 1A). Six hours after addition of either 20% FCS (Fig. 1A) or 100 ng of bFGF per ml (Fig. 1B), *gas6* mRNA is already down-regulated. After 6 h, its level is undetectable in the case of serum stimulation, while it steadily decreases to an undetectable level after bFGF addition.

The same Northern blot was normalized for the amount of total RNA with the *gapdh* cDNA probe, known to remain constant throughout the growth cycle. The percentage of cells in S phase, from each time point analyzed on the Northern blots, is shown in the histograms in Fig. 1.

Figure 2 shows the kinetics of *gas6* mRNA accumulation during growth arrest by serum starvation (Fig. 2A) or increased cell density (Fig. 2B). *gas6* mRNA is detectable after 12 to 24 h in medium containing low serum and reaches the highest level at 48 h (Fig. 2A). Normalization of RNA amount was similarly performed on the same blot with the

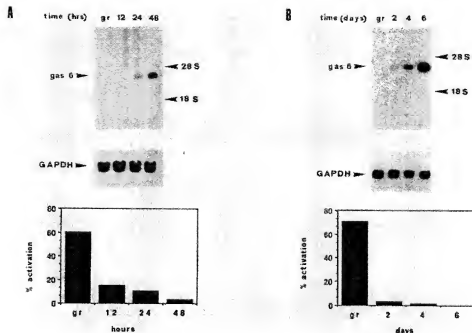


FIG. 2. Induction of *gas6* gene expression upon serum starvation and density-dependent inhibition in NIH 3T3 fibroblasts. (A) RNA was isolated from actively growing NIH 3T3 cells (gr = 24 h after seeding in 10% FCS) and at the indicated times after serum starvation in 0.5% FCS; (B) RNA was isolated from actively growing NIH 3T3 cells (gr = 24 h after seeding in 10% FCS) and at every 2 days before seeding with fresh culture medium (containing 10% FCS). Equal amounts (20 μ g) of total RNA were analyzed by Northern blotting. The same blots were also probed with the *gapdh* cDNA probe. The histograms show the relative level of DNA synthesis for each time point analyzed on the Northern blots.

gapdh probe, and the percentage of cells in S phase was assessed by BrdU incorporation at each time point (shown in the histograms).

To analyze the expression of *gas6* mRNA in relation to growth arrest induced by density-dependent inhibition, NIH 3T3 cells were seeded in 10% FCS, and the medium was changed every 2 days. Figure 2B shows that *gas6* mRNA is significantly increased 2 days after seeding, and its accumulation levels off after 6 days. The same blot was normalized with *gapdh*. Under the same conditions, DNA synthesis was significantly decreased as soon as 2 days after seeding.

Mouse and human *gas6* cDNA sequences. The cDNA sequence of murine *gas6* is 2,556 nt long and encodes a protein of 673 amino acids (Gas6). Several full-length clones were analyzed both from the NIH 3T3 cDNA library and from a mouse kidney cDNA library, and all showed the same sequence and restriction pattern. The predicted protein sequence of murine *gas6* was compared against the entire protein sequence data bank, using FastDB (1). A significant homology with bovine and human vitamin K-dependent protein S (15, 42) emerged, with 43% identity between the 673 residues of murine Gas6 and the 677 residues of human protein S. The residue identity is 42% between murine Gas6 and the 676 residues of bovine protein S. To assess whether Gas6 was the murine homolog of human protein S or a related but different protein, we screened a human lung fibroblast cDNA library with murine *gas6*. A partial cDNA clone representing the human homolog was isolated and used to screen a HeLa cDNA library. A full-length cDNA clone was thus isolated and sequenced. The clone analyzed is 2,461 nt long and encodes a protein of 678 amino acids

with 81% residue identity to murine Gas6 and 44% amino acid identity to human protein S. This comparison shows that hGas6 is related to but different from protein S. This finding thus identifies a new member of the vitamin K-dependent family of proteins.

Both murine and human Gas6 primary structures were compared with that of human protein S. Figure 3 shows the alignments and for the sake of clarity is divided into four regions. Region A includes the amino terminus, which contains a very conserved hydrophobic stretch typically resembling a signal peptide. This structure is consistent with protein S being a secreted protein and suggests a similar fate for Gas6. This region also contains the γ -carboxyglutamic acid (Gla) domain (16, 42) of protein S fully maintained in the human protein S (16). The Gla domain, present within the family of vitamin K-dependent proteins, is required for the calcium-dependent phospholipid binding that mediates the interaction of these proteins with cellular membranes (28, 59). A similar Gla domain-dependent interaction of Gas6 with cellular membranes may indicate a strict requirement for its compartmentalization in the regulation of a protease cascade (44). The short region B is known as the thrombin-sensitive segment of protein S (13). A Leu-Arg-Ser span represents the two thrombin cleavage sites in protein S. The comparable amino acid spans are Met-Arg-Lys and Phe-Ala-Lys in murine Gas6 or Ile-Gln-Lys and Phe-Ala-Thr in hGas6. The missing consensus may suggest that the Gas6 region B is not susceptible to the proteolytic attack by thrombin required for the negative feedback loop of the

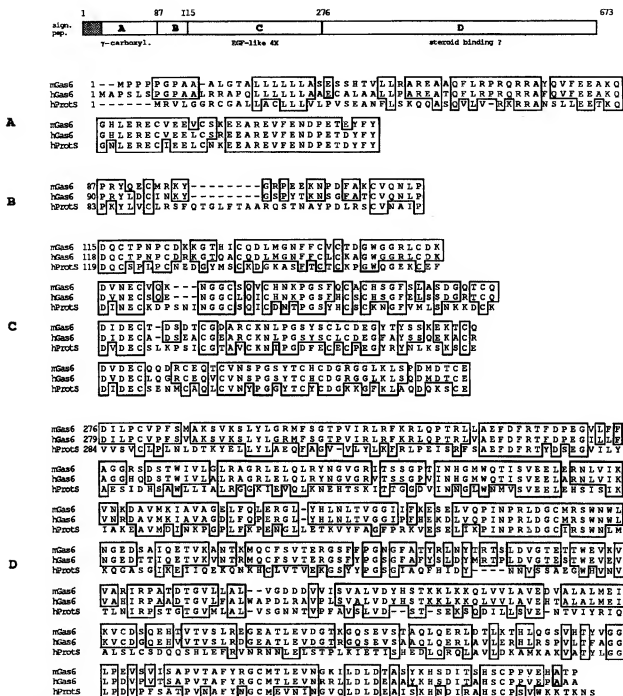


FIG. 3. Analysis of the *gas6* cDNA-encoded protein. The diagram at the top shows the overall organization of the predicted Gas6 sequence and the relative sizes of the four regions in the protein. Comparison of the predicted amino acid sequences of mGas6, hGas6, and human protein S is shown below. A, B, C, and D refer to the four regions present in these proteins (see Results for details).

coagulation cascade (13, 60). It is noteworthy that this region presents the lowest degree of homology to protein S (16% identity) relative to the other regions.

Region C includes four epidermal growth factor (EGF)-

like repeats (21, 67), each containing six cysteines. A consensus sequence for β -hydroxylation of Asp and Asn residues is contained in each of these domains, as is the case for protein S (56). Hydroxylated Asp and Asn play a role in the

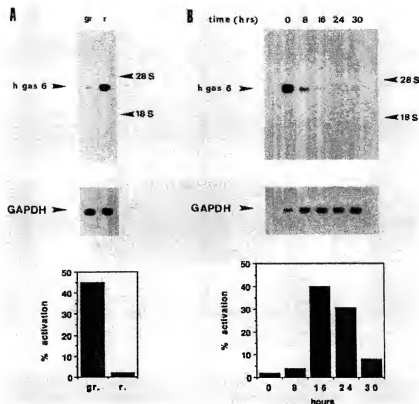


FIG. 4. Analysis of *hgas6* mRNA expression. RNA was extracted from actively growing and 72-h serum-starved human IMR90 fibroblasts (A) or serum-starved cells at different times after 20% FCS serum induction (B). The same Northern blots were probed with *gapdh*. The histograms show the relative percentage of DNA synthesis for each time point.

high-affinity binding of Ca^{2+} , as recently shown by nuclear magnetic resonance spectroscopy for the first EGF-like domain of factor IX (35), and are involved in high-affinity protein-protein interactions (51). The first EGF-like domain of *Gas6* is the one possessing the lowest homology (42% amino acid identity) to the corresponding domain of human protein S relative to the other EGF domains (domains II [48% identity], III [45% identity], and IV [51% identity]).

The C-terminal region D is the most extensive in length and, like human protein S, does not show any resemblance to serine proteases. As is the case for human protein S, region D of *Gas6* shows similarity to the human sex hormone-binding protein (SHBP) and rat androgen-binding proteins (2, 30). The portion of *hGas6* containing the highest amino acid identity (30%) and the minimum number of gaps with human SHBP is included within amino acids 315 to 457. This similarity suggests that region D could be involved in steroid hormone binding. Other ECM components, including laminin A chain and agrin, show homology to SHBP within the same region as *Gas6* (4). However, there are no experimental data to indicate that these proteins are capable of binding steroid-derived molecules. The carboxy-terminal part of *mGas6* has two potential glycosylation sites at positions 417 and 488, the first being conserved also in *hGas6* very close to the positions of similar potential glycosylation sites found in the other two species of protein S. The domain that interacts with the C4b-binding protein (C4BP) (17), a high-molecular-weight plasma protein involved in activation

of the classical pathway of the complement system (27, 32), is thought to lie within region D of protein S. In particular, the peptide (Gly-605-Ile-614) proximal to its C terminus specifically competes for interaction with C4BP (64). The C4BP domain involved in the interaction with protein S lies within a short consensus repeat (SCR) domain (12) of the α chain and possibly of the β chain of C4BP (38, 39). The corresponding region of *Gas6* shows 50% amino acid identity to the peptide (Gly-605-Ile-614) of protein S.

Regulation of *hgas6* in human fibroblast growth arrest and during the cell cycle. The human cDNA clone of *gas6* was used as a probe in Northern analysis of total RNA extracted from human IMR90 fibroblasts cultured under different growth conditions. Figure 4A shows *hgas6* expression in growing and serum-starved IMR90 human fibroblasts. The level of *hgas6* is significantly increased at growth arrest. Figure 4B shows *hgas6* expression during a synchronous cell cycle reinduction of serum-starved IMR90 fibroblasts. *hgas6* mRNA level is significantly decreased at 8 h after serum addition, reaching its lowest level at 16 h, which is maintained thereafter. The same Northern blots were probed with *gapdh* cDNA (histograms in Fig. 4), and the percentage of cells in S phase was also determined. Altogether, these results indicate that the expression of *hgas6* mRNA in IMR90 human fibroblasts is similar to that described for *mgas6* in NIH 3T3 mouse fibroblasts.

Analysis of *gas6* mRNA expression in tissues and cell lines. Total RNA isolated from different human and mouse tissues

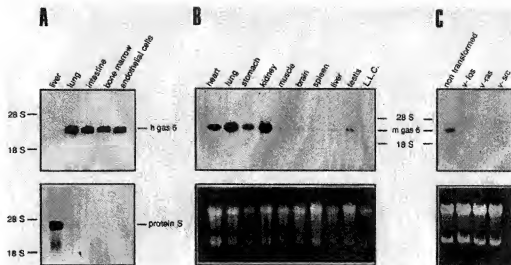


FIG. 5. *gas6* mRNA expression in various tissues and cells. (A) *gas6* mRNA expression in human tissues. Equal amounts (20 μ g) of total RNA, as estimated by ethidium bromide staining, were analyzed by Northern blotting. The upper panel shows the expression of *gas6*; the same Northern blot was hybridized with the human protein S probe (lower panel). (B) *gas6* mRNA expression in various mouse tissues. Equal amounts (20 μ g) of total RNA were analyzed by Northern blotting; the ethidium bromide staining is shown in the lower panel. L.L.C., Lewis lung carcinoma. (C) *gas6* mRNA expression in nontransformed and single-oncogene-transformed NIH 3T3 cells previously grown in 0.5% FCS for 48 h. Equal amounts (20 μ g) of total RNA were analyzed by Northern blotting; the upper panel shows *gas6* mRNA expression; ethidium bromide staining is shown in the lower panel.

was analyzed for *gas6* mRNA expression by Northern blotting. Comparable amounts of total RNA, as determined by ethidium bromide staining, were analyzed. Figure 5A shows that *gas6* mRNA (with a relative size of 2.6 kb) is expressed in all tissues analyzed at comparable levels except in liver, where it is apparently undetectable. The same Northern blot was also probed with human protein S cDNA (kindly provided by B. Dahlbäck). The lower panel of Fig. 5A shows that protein S mRNA (relative size of 3.5 kb) is expressed in liver and at a very low level (almost undetectable) in the other tissues analyzed. Figure 5B shows an analysis of *gas6* mRNA expression in several mouse tissues, using equal relative amounts of total RNA, as determined by ethidium bromide staining (lower panel). *gas6* seems to be expressed in many tissues analyzed, with higher levels in heart, lung, stomach, and kidney. RNA from a lung tumor (Lewis lung carcinoma) showed no detectable *gas6* mRNA. Since *gas6* expression is dramatically reduced in at least one tumor in vivo, we analyzed *gas6* mRNA expression in various NIH 3T3 cell lines transformed by single oncogenes. These lines were grown in low serum (0.5% FCS) for 48 h, condition that promotes *gas6* mRNA expression in nontransformed NIH 3T3 cells. Figure 5C shows that under these conditions, the normal NIH 3T3 cells express a significant level of *gas6* mRNA, while the single-oncogene-transformed lines do not present a detectable level of *gas6*.

hGas6 biosynthesis under different growth conditions. An antibody to the hGas6 (amino acids 359 to 551) expressed in *E. coli* was affinity purified as described in Materials and Methods. To confirm that this antibody specifically recognizes the hGas6 product, we first immunoprecipitated the hGas6 primary in vitro translation product. The in vitro-transcribed hGas6 RNA was used to program a rabbit reticulocyte extract; the protein obtained (Fig. 6A, lane 1) has an apparent molecular mass of 75 kDa, as expected from the cDNA sequence. Lane 2 represents the mock control in

which no RNA was added. When the total hGas6 translation was immunoprecipitated with the anti-hGas6 antibody, the same band representing hGas6 is visible (lane 4), while no band is detected when preimmune serum was used (lane 3). The hGas6 immunoprecipitated both from cell extracts (lane 5) and from conditioned medium (lane 6) has an apparent molecular weight similar to that of the in vitro primary translation product. The presence of hGas6 in conditioned medium indicates that it is secreted, as suggested from the presence of a signal sequence in cDNA sequence analysis. We therefore immunoprecipitated biosynthetically labeled hGas6 from exponentially growing and serum-starved cells. The same number of trichloroacetic acid-precipitable counts was used in the following comparative experiments.

As shown in Fig. 6B, growth arrest induced by low serum increases the level of hGas6 both in cell extracts (lane 2) and in the conditioned medium (lane 5) relative to the level of the protein synthesized by exponentially growing cells (lanes 1 and 4). To characterize hGas6 synthesis during the G₀-S transition, 20% FCS was added for 8 h to serum-starved cells and [³⁵S]methionine was added for a further 3-h labeling period. The amount of hGas6 immunoprecipitated both from cell extracts (lane 3) and from culture medium (lane 6) is clearly decreased relative to the result for serum-starved cells. Thus, the levels of hGas6 protein are consistent with mRNA expression.

DISCUSSION

It is becoming increasingly clear that cell proliferation is modulated by a complex network of interactions mediated by extracellular, cell-matrix, and cell-cell adhesion factors (66). A potential approach to identify negative control elements has been the isolation and characterization of genes expressed during growth arrest (*gas*) (6, 43, 54). We have recently shown that one of these genes, *gas1*, is directly

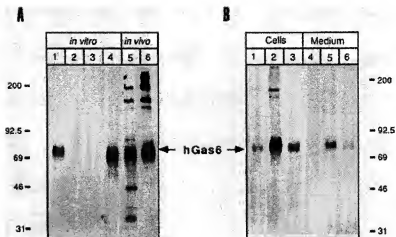


FIG. 6. Immunoprecipitation analysis of hGas6. (A) Shown are in vitro translation of hGas6 mRNA (lane 1), mock translation (lane 2), immunoprecipitation of the in vitro-translated hGas6 mRNA by using preimmune antiserum (lane 3) and anti-hGas6 affinity-purified antibodies (lane 4), and immunoprecipitation of hGas6 from serum-starved cellular lysates of IMR90 fibroblasts (lane 5) and the respective culture medium (lane 6) after [35 S]methionine in vivo labeling for 14 h. (B) Immunoprecipitation analysis of hGas6 from IMR90 fibroblasts. Conditions were as follows: exponentially growing, 24 h after seeding in 10% FCS, and 3 h of [35 S]methionine labeling (lanes 1 [cellular lysate] and 4 [culture medium]), serum starved, 72 h of 0.5% FCS incubation and 3 h of [35 S]methionine labeling (lanes 2 [cellular lysate] and 5 [culture medium]), and serum starved after 8 h of incubation with 20% FCS and an additional 3 h of [35 S]methionine labeling (lanes 3 [cellular lysate] and 6 [culture medium]). Equal numbers of trichloroacetic acid-precipitable counts from the respective cellular lysates (lanes 1 to 3) or culture medium (lanes 4 to 6) were processed for immunoprecipitation.

involved in growth suppression (19). In this report, we have described the structure and expression of mouse and human *gas6* cDNAs. *gas6* expression is induced after serum starvation but decreases dramatically after induction to reenter the cell cycle both in mouse NIH 3T3 cells and in human IMR90 fibroblasts. By making use of a polyclonal antibody raised against hGas6, we have also shown that the protein is secreted and that its biosynthetic level fully reflects mRNA expression.

Sequence comparisons of both murine and human cDNA have revealed that *gas6* is a new member of the family of vitamin K-dependent proteins homologous to protein S. Protein S acts as a molecular scatchen (52) in both the blood coagulation and complement cascades. During blood clotting, protein S interacts with activated protein C, and the complex catalyzes the proteolytic inactivation of factors Va and VIIIa, which are involved in thrombin activation (62, 63, 65). This protein thus acts as an important negative regulator of the blood-clotting cascade (23, 24). The involvement of protein S in the complement cascade is demonstrated by the finding that approximately half of the protein S in human plasma is bound to C4BP (17). Although its role in the complement cascade is not clear, protein S represents a unique link between the two protease pathways that regulate the complement and coagulation systems (37).

As shown by sequence comparison, the regions with the highest homology between Gas6 and protein S are region A (Gla domain) and region C (EGF domain). Region B is the most divergent, having lost the thrombin-sensitive sites. It has been proposed that the region of protein S involved in the interaction with activated protein C includes the first EGF domain (which in Gas6 bears the lowest homology with protein S relative to the four EGF-like domains) and the thrombin-sensitive segment (14) (whose corresponding region B in Gas6 shows the lowest homology relative to all the other regions). This finding suggests that a putative interac-

tion of Gas6 with protein C, and consequently a specific involvement in the control of the coagulation cascade, is rather unlikely.

On the basis of the known involvement of protein S in the complement cascade, an interaction of Gas6 with C4BP also appears unlikely, since its putative binding region has only 50% identity to the corresponding region of protein S. However, because the target of this interaction appears to reside in the SCR domains (also known as complement-related protein domains) of C4BP, Gas6 might interact with other SCR-containing proteins. SCR domains are present in the rapidly expanding family of LEC-CAM adhesion molecules (57) as well as in some proteoglycans such as versican (68).

We can speculate that the putative function of Gas6 is likely to be different from the more restricted role of protein S in the blood coagulation and complement cascades. This would not, however, exclude the possibility that protein S itself, given the functional promiscuity in protease networks, has other functions. In fact, protein S has recently been reported to be a potent mitogen for smooth muscle cells (29). In the light of this finding, *gas6* also might have a role in cell proliferation. This hypothesis is supported by the fact that its expression seems to be less restricted than that of human protein S, being detectable in many human and mouse tissues. However, it remains to be determined whether *gas6* is expressed in different cell types or its expression is restricted to a subset of cells (endothelial cells) ubiquitously present in the tissues analyzed.

It is generally accepted that a complex equilibrium of interactions links ECM and the cell surface, both of which play an active role by focalizing growth factors, proteases, and protease inhibitors close to the site of action. We hypothesize that Gas6 may be a component of a protease cascade involved in growth regulation. The rationale behind this hypothesis originates from the strict dependence of its

expression on growth arrest. The relief from this dependence, as is the case for transformed cells *in vitro* or *in vivo*, should be the necessary consequence.

ACKNOWLEDGMENTS

We thank S. Bottega for skillful technical assistance in the production and purification of anti-Gas2 antibodies and P. Marcanelli for help in sequencing the *iguos6*, part of which was done during a short stage at the EMBL DNA Sequences Facility. We thank W. Anzorge and N. Hewitt for kind hospitality and technical advice. We thank J. Stenflo and B. Dahlbäck for kindly providing human protein S, its cDNA, and antibodies. We are indebted to P. Mignatti (University of Pavia) for carefully reading and suggesting corrections to the manuscript. We warmly thank L. Philipson for continuous encouragement and advice during this work.

This investigation was generously supported by grants from the Associazione Italiana per la Ricerca sul Cancro (AIRC)—Progetto Speciale Oncosoppressori, CNR P.F.F. Ingegneria Genetica, and CNR-ACRO to C.S. C.B. is an AIRC fellow.

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